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ECTODERMAL LESIONS PRODUCED BY THE VIRUS OF ROUS SARCOMA.

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THE use of the developing chick embryo and its membranes in the study of filtrable viruses originated with the investigations of Murphy and Rous (1912), who showed that when the embryonic membranes or the embryo itself were inoculated with the agent of Rous Sarcoma No. 1, tumours arose at the sites of puncture. Such tumours were, in their experience, invariably sarcomata, arising only when the inoculum came in contact with mesodermal tissue. Since they pierced the membranes with a hollow needle containing tumour suspension or filtrate, mesodermal tissues were necessarily always exposed to the action of the virus. When the inoculum was deposited in the extra-embryonic body-cavity, which is lined by mesoderm, discrete focal proliferations appeared, which were evidence of the particulate nature of the agent. In the experiments here described, the standard technique of this laboratory was employed, in which the inoculum is deposited on the intact ectodermal surface of the chorio-allantois. Using this method, inoculation of cell-free filtrates of the virus of Rous sarcoma gives rise to focal proliferations of ectodermal cells resembling in many respects the lesions produced by other viruses on the chorio-allantois, but showing characteristic differences.

METHODS.

The virus was kindly supplied by Dr. C. H. Andrewes as a tumour desiccate. On receipt, two serial passages were made in Plymouth Rock hens and two further passages in White Leghorns. The incubation period fell from 21 days in the first passage to 10 days in the fourth passage. The tumour of the 4th passage was macroscopically and microscopically a typical No. 1 tumour. An emulsion of this tumour containing living cells was used as the initial inoculum of the chorio-allantois. Subsequent passages were made solely

with egg membrane material, obtained by grinding a membrane bearing lesions with powdered silica, emulsifying in 5 c.c. nutrient broth pH 7·6, centrifuging 5 to 10 minutes at 2000 r.p.m. in the horizontal centrifuge, and using the supernatant fluid for inoculation. In some experiments filtrates of such supernatants through Gradocol membranes (A.P.D. approximate 0·7μ) were employed.

The technique of inoculation has been described in several studies from this laboratory, and a detailed description has recently been given by Burnet (1936). The essential point of the technique is the establishment of an artificial air space, thus providing a flat sheet of chorio-allantois with an intact ectodermal surface to receive the inoculum. White Leghorn eggs were used throughout. As routine they were inoculated on the 12th day of incubation, and the membranes removed for examination 7 days later. Occasionally, for specific purposes, eggs incubated for 10 or 11 days were used, and to follow the development of the lesions, membranes have been removed at various intervals from the 3rd to the 10th day following inoculation.

RESULTS.

(a) *Lesions Appearing from the Primary Inoculation.*

As has been stated, the initial inoculation was made with a fresh emulsion of an active fowl tumour, which contained living cells. In eggs opened at the 7th day there were present large central tumours, dependent from the under-surface of the membrane, exactly resembling those described and figured by Murphy and Rous (1912). Histologically also the appearances coincided with the illustrations of these authors.

In addition to these large central tumours, however, some small discrete pearly focal thickenings were noted, irregularly scattered on the membranes about the periphery of the central mass. A piece of chorio-allantois bearing several such nodules was excised, and an emulsion used to inoculate further eggs. Similar discrete lesions were noted in membranes removed from the 6th day onwards. In this second passage no central tumours appeared. A series of 13 passages was made, similar lesions, usually some 50 on each membrane, appearing. Similar passages have been initiated with dried material of the original tumour, and from fresh material from Rous No. 1 sarcomata arising from inoculation of egg passage material. The present series of inoculation has reached its 30th passage. With later passages the titre of the membrane emulsions is high, and the supernatant, prepared as described above, is usually infective at a dilution of 10^{-4}, using a standard inoculum of 0·05 c.c.

On histological examination these superficial nodules proved to be focal ectodermal proliferations.

(b) *Macroscopic Appearances of the Lesions.*

Membranes examined in the first 3 days following inoculation show no specific changes. There are the occasional non-specific lesions incidental to the method of inoculation which are readily distinguishable from the uniform

focal lesions produced by viruses pathogenic for the chorio-allantois. Recognizable lesions appear from the 4th day onwards, increase in size with incubation, and are of convenient size for enumeration, with their characteristic appearances fully developed by the 7th day. By the 8th day following inoculation (the 20th day of incubation) the chick is about to hatch and frequently has pierced the inoculated area with its beak, tearing a circular hole which renders the membrane unsuitable for examination.

The lesions as they appear on typical 7-day membranes are illustrated in Fig. 1. They are flattened circular pearly opacities, ranging in diameter from 0·5–2 mm. The average size of the lesions is fairly uniform on a single membrane, but varies slightly with different membranes receiving similar inocula. The macroscopic appearances are quite distinctive, and the lesions could not be confused with any non-specific lesion seen in this laboratory or with those produced by any of the viruses studied by this technique (vaccinia, fowl-pox, canary-pox, laryngo-tracheitis, ectromelia, psittacosis, myxomatosis, Newcastle disease, louping ill, influenza). This distinctive appearance is correlated with their slow development. The lesions of all the above-mentioned viruses are recognizable within 1 to 3 days of inoculation, and the membranes are removed for examination on the 2nd or 3rd day, in contrast to the 7 days required for comparable development of the Rous lesion. Discrete lesions appear only if dilute inocula are used. With increasing concentration confluent lesions arise (Fig. 2); with very concentrated supernatants central tumours (sarcomata) appear (Fig. 3).

(c) *Histology of the Lesions.*

Histological examination shows these lesions to be focal proliferations of ectodermal cells. The earliest distinctive changes are seen in membranes removed at the 4th day following inoculation. These show in certain limited areas a differentiation of the ectoderm into two strata—an external layer of flattened cornified cells, staining deeply with eosin, and an internal layer of rounded cells, staining much less intensely. A very early stage of this cellular proliferation is seen in Fig. 4. There is some increase in thickness of the mesodermal layer, without evidence of proliferation of the mesodermal cells. The entodermal epithelium is unaffected. A moderate number of leucocytes may be found in the mesodermal layer, particularly beneath the areas of proliferation, but such leucocytic infiltration is minimal, and in some sections absent. The changes, apart from the focal areas of proliferating basal cells, are practically indistinguishable from those resulting when the dropped membrane is inoculated with sterile broth. In such cases, however, the traumatic reaction has usually subsided by the 3rd day. During the subsequent days the ectodermal cells of the lesions continue to proliferate. The extension is at first mainly laterally and slightly towards the free surface, so that vertical section through the centre of such an area shows the proliferating cells arranged in a semi-ellipse with a horizontal base (Fig. 5). Mitotic figures are common. The capillaries lying just below the basal layer of ectoderm are gradually included in the mass. The proliferating cells appear to grow round them, the capillary endothelial cells are normal, and the capillaries themselves are not dilated. Sections taken at a later stage on the 6th and

7th days (Fig. 6) show further increase in size. The proliferating cells no longer spread mainly laterally and toward the external surface, but actively invade the mesoderm. The appearances suggest that at the central portion of the mesodermal aspect of the mass ectodermal cells have burst some restraining barrier, and on reaching the loose mesodermal tissue have continued to proliferate. By this time cells in the main nodule differ from those in the early stages of development. They are very large, and many contain ingested leucocytes or erythrocytes. The nuclei are large, pale and vesicular with prominent nucleoli. Mitotic figures are not so common, particularly in the cells in the centre of the mass. The protoplasm at the periphery stains strongly with eosin, and takes a bright red with Mallory's stain, suggesting cornification. The cells which are invading the mesoderm are of a more active anaplastic type, with frequent mitotic figures, large nuclei and less abundant protoplasm staining homogeneously. There appears to be little or no inflammatory reaction to this intense cellular proliferation. Although there are phagocytosed leucocytes present in the cells, they are not very abundant, and probably are derived from the leucocytes present in the capillaries invaded by the mass rather than by emigration from unaffected vessels. The mesodermal cells adjoining the epithelial masses appear normal.

The process of invasion continues until the mesoderm may be almost entirely replaced by the invading cells (Fig. 7). In spite of this, practically all embryos hatch out, and the chicks survive, usually for about three weeks, eventually dying with multiple tumours in the liver and/or lung, which are typical sarcomata. No macroscopic lesions have been found in embryos removed at the 7th or 8th day, nor have any lesions been observed in membranes other than the chorio-allantois. In certain circumstances, with egg passage material, connective-tissue proliferation does occur in the mesodermal layer. This occurs only when supernatants of high titre are used, giving rise to confluent lesions, never when the dilution of the inoculum is such that only discrete lesions appear. Such mesodermal lesions appear in the central portion of the membrane. This is the site where non-specific ulcers appear in membranes inoculated with any virus or with virus-free material (Burnet, 1936 ; Keogh 1936). Such traumatic ulcers are usually healed by the 3rd or 4th day, whereas when strong egg-passage Rous virus is used as inoculum there is a continuous growth of the central lesion up to the time of hatching. This is apparently due to direct infection of mesodermal cells. The probable explanation is that during the process of preparation of the artificial airspace, when a tear is made in the shell membrane, a few ectodermal cells are damaged or displaced, so that a small area of mesoderm is exposed to infection. With dilute inocula the chance of an infective particle settling on this small area is remote, but the probability increases with stronger inocula, and when the conditions are such as to give confluent lesions, infection of this central spot of exposed mesoderm is the rule. Such lesions when sectioned show either a combination of ectodermal and mesodermal or purely mesodermal proliferation. Fig. 8 shows a section through such a lesion. There is marked proliferation of mesodermal cells. This might be interpreted as an inflammatory reaction to the invading ectoderm, except that in membranes inoculated with dilute virus suspensions where the ectodermal proliferation and invasion are just as marked,

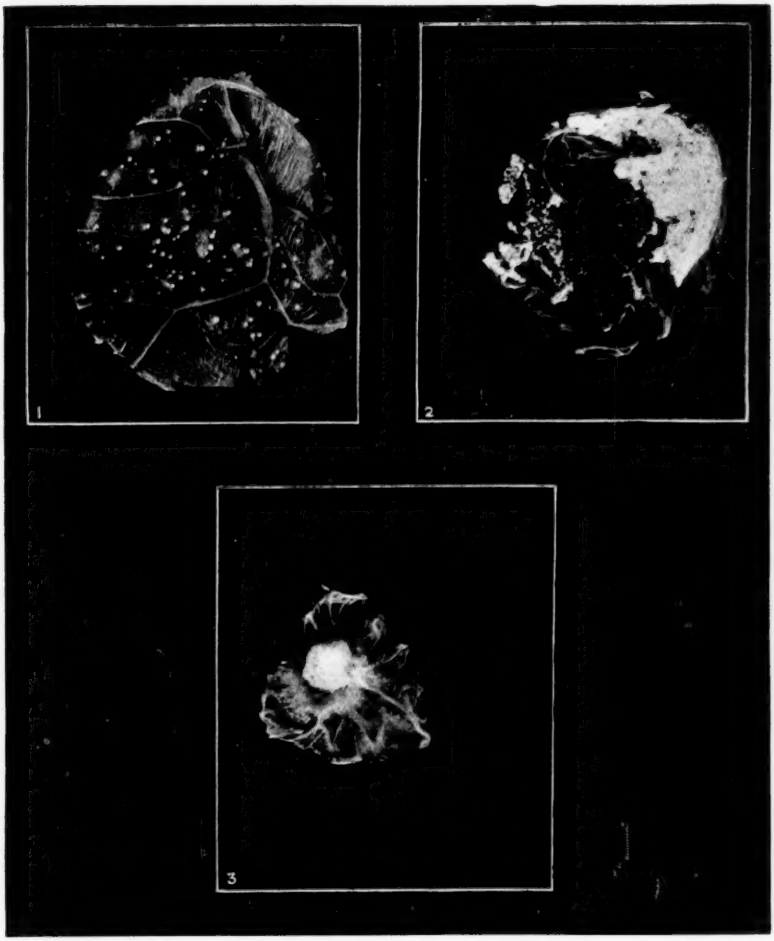

FIG. 1.—Chorio-allantoic membrane showing focal ectodermal proliferations produced by dilute suspension of Rous virus. Natural size.

FIG. 2.—Semi-confluent mixed ectodermal and mesodermal proliferation produced by more concentrated suspensions. (Fig. 8 shows histology of a similar membrane.) Natural size.

FIG. 3.—Central mesoblastic tumour (sarcoma) produced by very concentrated suspensions. Natural size.

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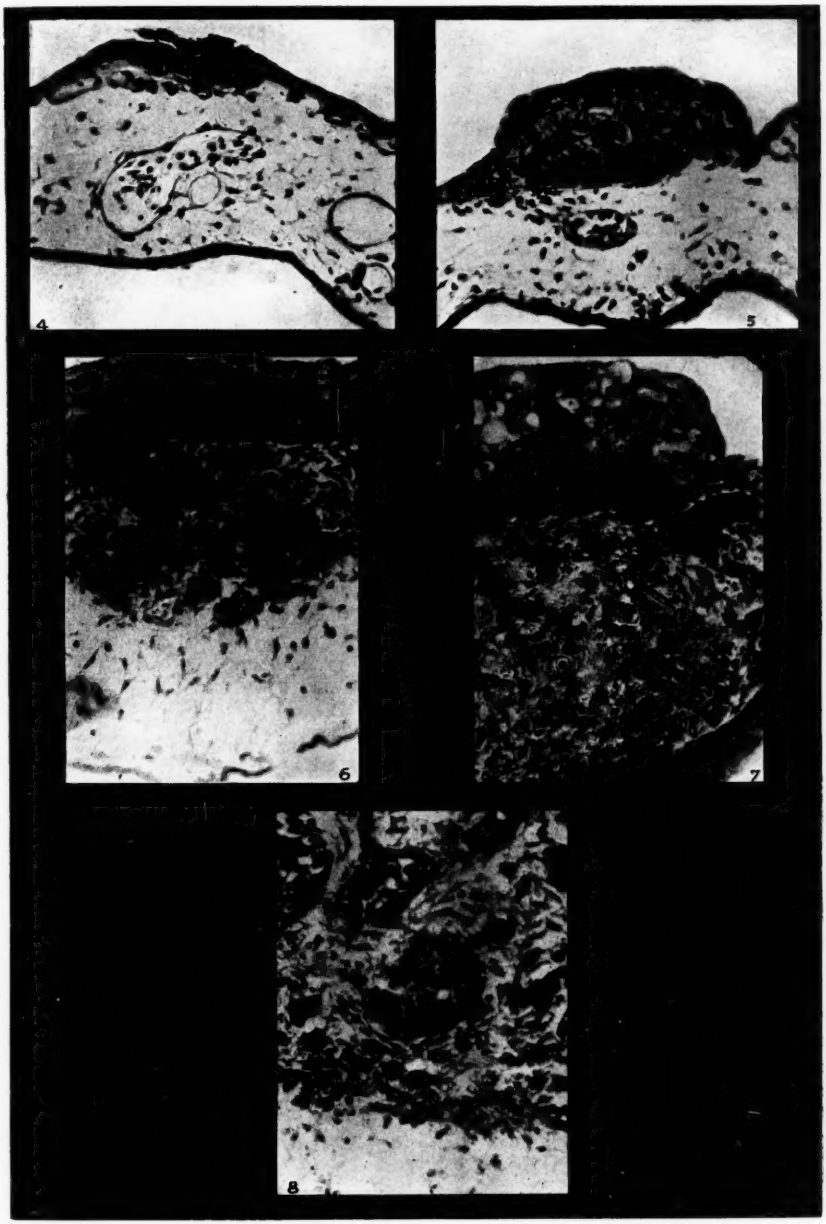

FIG. 4.—Early ectodermal lesion. Membrane removed 4 days after inoculation. × 400.

FIG. 5.—Ectodermal lesion. Membrane removed 6 days after inoculation. × 400.

FIG. 6.—Ectodermal lesion 7 days after inoculation, showing invasion of mesoderm. × 400.

FIG. 7.—Eight-day lesion, showing complete replacement of mesoderm by proliferating ectodermal cells. × 400.

FIG. 8.—Seven-day membrane. Mixed ectodermal and mesodermal proliferation. Note sharp differentiation of proliferating cells from unaffected mesoderm. × 400.

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there is no such reaction. Further, the histology of the proliferating mesoderm is that of the nodules produced by deliberate infection of the mesoderm as figured by Murphy and Rous (1912), and of the practically pure mesodermal proliferations which may arise in response to inoculations of very concentrated extracts either of active fowl sarcomata or of egg-passage material (Fig. 3). In addition there is a definite boundary to the area of proliferating mesoderm, rendered visible by slightly different staining properties of the affected area, and by a border of compressed normal mesodermal tissue.

(d) *Results of Inoculation of Fowls with Egg-passage Virus.*

When it was found that the Rous agent was capable of initiating lesions in the chorio-allantoic ectoderm, it was thought that some measure of adaptation might be attained by passage, and that corresponding epithelial proliferations might be obtained in the fowl.

With that object fowls were inoculated in the feather follicles with egg-passage virus of the 6th passage. No lesions appeared in 2 fowls within 3 weeks of inoculation. It was decided to use these fowls for attempted production of immune sera to the virus. Each was inoculated intraperitoneally with 2 c.c. of undiluted egg-passage virus. Two days later small nodules appeared at the sites of the inoculated follicles, which progressively enlarged. A biopsy on one of the tumours, however, showed it to be a typical sarcoma, and both fowls succumbed later with multiple tumours, all sarcomata. Intradermal inoculations were made with egg-passage virus derived from membranes bearing only discrete ectodermal lesions. Sarcomata arose at the sites of inoculation in 5 out of 8 fowls, 3 showing no lesions. These sarcomata, as judged by macroscopic and histological appearances, were typical Rous sarcomata.

Three fowls were inoculated in the subareolar tissue of the breast with finely minced ectodermal lesions from membranes of the 13th passage. After an incubation period of 6 days nodules arose at the sites of inoculation, which rapidly enlarged. Biopsy of one tumour showed it to be a sarcoma, and the 3 fowls eventually succumbed, again with typical primary and secondary Rous sarcomata. It is to be noted that one of these fowls had on two previous occasions failed to react to intradermal inoculation of egg-passage virus which produced sarcomata in other fowls. There was a flaw in this experiment in so far as the attempted production of an epithelial tumour by multiplication of introduced, presumably virus-infected embryonic ectodermal cells was concerned. The embryonic cells were from White Leghorn eggs, but were implanted in Barred Plymouth Rock fowls.

Intramuscular injections of 20th passage egg virus into White Leghorn chicks resulted in the production of sarcomata at the sites of inoculation. The lowest dose tested produced a tumour. It was 0·05 c.c. of a 1 : 10,000 dilution of the supernatant of suspension obtained by grinding a membrane (weight approximately 2 g.) in 5 c.c. of broth.

It will be noted that in both the chick and fowl the only tumours obtained were typical sarcomata. It is hoped to pursue these experiments on fowls after prolonged egg passage of the virus.

(e) *Titration of Suspensions by Enumeration of the Lesions.*

The number of lesions appearing on the membranes is directly proportional to the concentration of the inoculum over the range in which discrete foci appear. Table I records a typical experiment.

TABLE I.

Dilution.	Lesions per membrane.	Average.
1 : 10	340, 148, 130, (0)	206
1 : 100	27, 16, 13, 12	17
1 : 1000	4, 1, 1, 0	1·5

(0) = Membrane with no foci, omitted for calculation of average.

Not all titrations produce such regular results. There is almost always a varying number of membranes showing few or no lesions, irrespective of the concentration of the inoculum, usually not more than 10 p.c. of those inoculated, but varying up to 50 p.c. in some experiments. The possibility that this poor response is due to the presence in such eggs of antibodies to the virus derived from the mother cannot be excluded. It seems more probable, however, that it arises from technical difficulties in inoculation. A similar phenomenon, though to a less marked degree, is noted with other viruses. The unresponsive membranes are often unduly dry or extremely œdematous, and such specific lesions as do appear are present on the periphery of the chorio-allantois, where it has not been subjected to the trauma of detachment from the shell membrane. There are further difficulties in these titrations, arising from the ready spontaneous inactivation of the virus even at ice-box temperature, particularly in dilute suspension. It was usually found that the dilutions inoculated first gave proportionately higher counts. Inactivation proceeds more rapidly at higher temperatures. Table II records the course of inactivation of the virus at 57° C.

TABLE II.

Dilution.	Time of inactivation at 57° C. in minutes.	Lesions per membrane.	Calculated titre of undiluted suspension per c.c.
1 : 50	0	243, 142, 119, 45	$1{\cdot}4 \times 10^5$
1 : 5	5	320, 193, 94, 78	$2{\cdot}2 \times 10^4$
1 : 5	10	59, 38, 5, 4	$2{\cdot}6 \times 10^3$
1 : 5	15	2, 0, 0, 0	$0{\cdot}5 \times 10^2$

Inoculum 0·05 c.c. of stated dilution.

It is seen that 15 minutes at 57° C. has almost completely inactivated a suspension of high initial titre, reducing it between 1000 and 10,000 fold. This agrees with statements by various authors as to the rate of inactivation of the Rous No. 1 virus by heat.

Membranes kept in broth with a paraffin seal at 4° C. were still infective

at 6 weeks, but to a much less degree than fresh membranes. Glycerinated fowl tumour at 4° C. was slightly infective for the chorio-allantois at 3 weeks, but not at 8 weeks.

(f) *Attempts at Neutralization of the Virus with Sera of Various Types.*

The original incentive to this work was the hope that some means might be found of producing focal mesoblastic lesions in the membranes, so that the technique employed for the study of the immunology of other viruses (Burnet, Keogh and Lush, 1937) might be used to explore the immunology of the fowl tumours. When, unexpectedly, focal ectodermal lesions appeared, resembling in many ways those produced by the other viruses studied, it was hoped to study the effect of immune sera on the lesions. No satisfactory evidence of neutralization of the virus as evidenced by reduction in the number of lesions has been obtained with any serum tested. These include sera obtained from fowls bearing tumours and from fowls resistant to inoculation with suspensions of egg-passage virus which was infective for the chorio-allantois. In some experiments there was an apparently slight neutralization, but the effect was not reproducible and lay within the margin of experimental error. Rabbit sera obtained by immunization with egg-passage virus, with fowl tumour suspensions and with fowl erythrocytes were equally ineffective. These sera all contained anti-fowl precipitins, and gave marked precipitation with virus suspensions prepared from the chorio-allantois. In some experiments the antifowl sera actually appeared to have an activating effect as compared with normal rabbit serum, but the effect was inconstant, and no significance is attached to the results. No attempt has yet been made to produce sera in ducks, goats or horses—methods found effective by Gye and Purdy (1931)—nor has there been an opportunity to test sera from fowls bearing a slowly-growing fibro-sarcoma (Andrewes, 1931, 1932). It may be noted that sera of known activity against the viruses of fowl-pox and infectious laryngo-tracheitis of fowls also failed to influence the development of the Rous lesions.

DISCUSSION.

The evidence that these ectodermal lesions are actually caused by infection of the cells by the virus of Rous No. 1 sarcoma is strong. They appear only on membranes inoculated with extracts of such tumours, and have never been observed on control membranes inoculated with a wide range of tissue extracts, including the Fujinami tumour, and fowl lymphomatosis. They resemble no non-specific lesions seen in the laboratory, where over 20,000 eggs have been inoculated. After 17 serial passages, emulsions of egg material produced typical Rous tumours in susceptible fowls. The repeated production of lesions, by filtrates through Gradocol membranes eliminates any possibility of introduced living cells being responsible for them. The other possibility is that a virus capable of infecting ectoderm either present in the original material or picked up in one of the initial fowl passages is responsible for the lesions and that the Rous agent multiplies concurrently during serial transfer without producing macroscopic or microscopic evidence of its presence. Such a

hypothesis is almost incapable of disproof, and is mentioned only because of the *á priori* improbability of a virus hitherto considered specific for mesoderm causing lesions in ectodermal cells. One must further postulate that the hypothetical virus is able to initiate in the embryonic ectoderm processes which by histological criteria are malignant. None of the fowl viruses which are known to infect the chorio-allantois can be responsible for the lesions which are macroscopically different from those of fowl-pox and laryngo-tracheitis, and are not neutralized by sera active against these viruses, while fowl plague and Newcastle disease are rapidly lethal for the embryo.

In addition the responsible agent shows several of the known characteristics of the Rous virus, the instability on standing at room temperature, the range of heat inactivation and the difficulty of obtaining inactivating sera. The production of discrete lesions corresponding numerically to the dilution of the inoculum is proof of the particulate nature of the agent, while the infectivity of Gradocol filtrates shows that its size is in the virus range. Facilities are not available for exact determination of the particle size.

Features of interest in the development of the lesions are the origin in basal epithelium, the direction of spread of the multiplying cells, the delay in invasion of the mesoderm, the rapid extension when the mesoderm is invaded, the absence of stroma formation when discrete lesions are present, the apparent stroma reaction when very heavy infection takes place, and the absent or minimal inflammatory response to the invading cells.

These observations fall into line with the conception of Andrewes (1934) of a series of viruses graded according to the kind of pathological change induced, from necrosis to neoplasia. The virus responsible for these lesions evidently stimulates proliferation of the infected cells. There is no tendency to necrosis. When necrosis does occur, it is in the centre of large masses of proliferating cells, and is evidently due to lack of nutrition in the central parts of the tumour. In the absence of necrosis there is apparently no liberation of virus from the infected cells, since the adjacent susceptible mesoderm is not infected. The virus must multiply, since transmission in series is possible. The daughter-cells presumably carry the infecting virus with them when they divide. Study of the lesions leads to the belief that the discrete nodules are unicentric, originating from the unrestrained division of a single infected ectodermal cell.

The chorio-allantoic technique has possibilities in the investigation of avian tumours. Supernatant fluid from emulsions of infected membranes filters readily; the sticky material which hinders filtration of the Rous tumour is absent. Although the Fujinami tumour gave rise to no lesions on the chorio-allantois, it is possible that some of the other sarcomata may do so. It would be interesting to attempt propagation of an avian carcinoma by this method with a view to filtration studies. No neutralization has been demonstrated with the available sera, but the possibility of such an effect should not be abandoned until sera of known high activity in the fowl have been tested.

The chief interest of the present studies is the demonstration that the virus responsible for the production of a fowl sarcoma has the potentiality of infecting cells of ectodermal origin.

SUMMARY.

1. The virus of Rous sarcoma has been propagated for 30 generations on the chorio-allantois of the developing chick.

2. In this situation the virus gives rise to purely ectodermal focal lesions. When inoculated in dilute suspensions mixed mesodermal and ectodermal lesions arise.

3. The enumeration of the discrete lesions may be used as a method of titration of the virus.

4. Following inoculation into fowls of emulsions of egg-passage membranes bearing discrete ectodermal lesions, typical Rous sarcomata appear.

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THE EFFECT OF CATALASE ON THE RESPIRATION OF A FILTERABLE ORGANISM FROM SEWAGE.

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IN a previous paper (Pirie, 1937) the effect of hæmin on the respiration of a filter-passer isolated from sewage and described by Laidlaw and Elford (1936) was reported. By analogy with the stimulating effect of hæmin on the respiration of protozoa described by Lwoff (1934) it was suggested that hæmin was used by this sewage organism to synthesize cytochrome. This suggestion could not be proved owing to the small amount of organism available and its contamination with hæmin. A great difference between the protozoa and the sewage organism "C" was that 1·0γ hæmin per ml. measurably increased the respiration of the former, whereas 50γ per ml. was the smallest amount which had any effect on the respiration of the filter-passer. Laidlaw (personal communication) has since found that very much smaller amounts of catalase in the medium can replace hæmin and enable the organism to grow. The purpose of this note is to describe the effect of catalase on the respiration of the filter-passer and to consider its function.

TECHNIQUE.

The organism " C " was grown on 3 p.c. Difco proteose peptone in 0·9 p.c. NaCl with the addition of 50 mg. p.c. glucose and 3 p.c. Fildes (1920) blood digest, or an unknown amount of catalase sufficient to give maximum growth. After 24 hours' growth at 30° C. the organisms were centrifuged out of the medium and suspended in saline. The oxygen uptake of these suspensions was then measured in small Warburg manometers taking 1·0 ml. fluid only. All experiments were done at 30° C. Catalase was prepared from liver by the method of Keilin and Hartree (1936). To free the catalase from alcohol, which is present in the first phosphate extract from gelatinous calcium phosphate on which the catalase is adsorbed, a further two adsorptions and extractions were carried out.

EFFECT OF CATALASE.

Catalase acts in the same way as hæmin in enabling the organism " C " to oxidize glucose. No substrates other than monosaccharides have been found which can be oxidized with measurable speed. Quantitatively, catalase has a much greater effect than hæmin (Curve I). The total hæmin of one catalase preparation was roughly determined and found to be 50γ per ml. Catalase only represents part of this total, yet a concentration of 0·5γ total hæmin of this preparation measurably increased glucose oxidation. Since 50γ blood hæmin per ml. was required to show any effect, catalase is at least 100 times as effective as hæmin. The QO_2 for glucose oxidation in the presence of catalase was 58 (single observation). The QO_2 for glucose oxidation in the presence of hæmin lay between 30 and 50.

That catalase itself was responsible for the increase in oxidation was made probable by the fact that catalase preparations heated to 70° C. for $\frac{1}{2}$ hour, until only traces of catalase activity remained, failed to increase glucose breakdown (Curve II). Similarly, when *M*/500 hydroxylamine was added to the manometer cup, no oxidation took place (Curve III). Hydroxylamine in such a concentration strongly inhibits catalase activity. Peroxidase is unable to replace catalase.

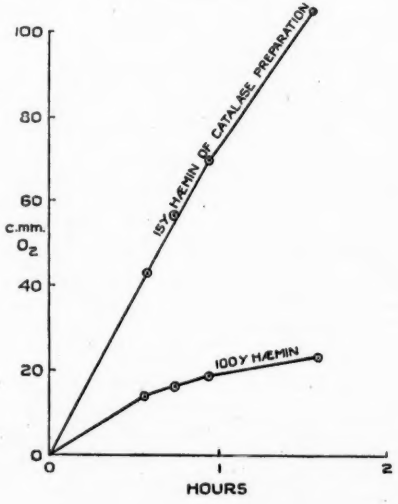
THE FUNCTION OF CATALASE.

The stimulating effect of catalase on bacterial growth has long been known and summaries are given in recent books ; see Topley and Wilson (1936) and Knight (1936). Organisms which are sensitive to hydrogen peroxide have been found to contain no catalase and *vice versa*. Obligate anaërobes do not contain catalase. The filter-passer " C " will grow anaërobically, or, very feebly, aërobically in the absence of catalase. We have to consider therefore whether catalase increases aërobic growth and, incidentally, the oxidation of glucose, by removing hydrogen peroxide, which may be destructive to the organism, or whether it fulfils some other function.

PRODUCTION OF HYDROGEN PEROXIDE.

Direct detection of hydrogen peroxide during the oxidation of glucose was impossible as no oxidation took place except in the presence of catalase. Indirect evidence for its production was obtained by showing that during oxidation of glucose in the presence of catalase a secondary oxidation of alcohol took place. Keilin and Hartree (1936) showed that during primary oxidations where H_2O_2 was produced, a secondary oxidation of alcohol occurred if catalase were also present.

CURVE I.



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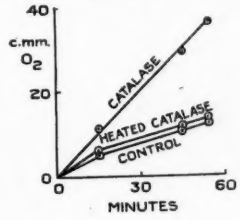
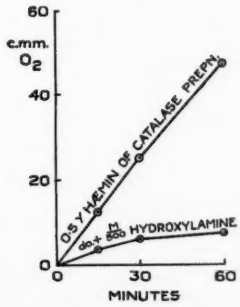


CURVE III.



CURVE I: *Catalytic effect of catalase compared to that of hæmin.*—Sedimented organisms from 64 ml. blood-glucose-peptone culture were made up in 2·5 ml. saline; 0·05 ml. *M* phosphate buffer pH 7·6, 0·05 ml. *M* glucose and 0·5 ml. suspension of organism "C" in each cup. 100 γ hæmin added to one cup and 15 γ total hæmin of catalase preparation added to the other. Final volume in cups 1·0 ml.

CURVE II: *Effect of heated catalase.*—Sedimented organism "C" from 32 ml. blood-glucose-peptone culture used. Phosphate and glucose concentrations as before; 0·05 ml. heated or unheated catalase added to the manometer cups. Catalase preparation was heated to 70° C. for 30 minutes.

CURVE III: *Effect of* M/500 *hydroxylamine on catalase effect.*—Sedimented organism "C" from 36 ml. blood-glucose-peptone culture used. Phosphate and glucose concentrations as before; 0·5 γ total hæmin of catalase preparation in both cups. *M*/500 hydroxylamine in one.

TABLE I.—*Secondary Oxidation of Alcohol.*

64·0 ml. of 24-hr. cultures in glucose-blood or glucose-catalase-peptone were centrifuged at 12,000 r.p.m. for 20 minutes. The sedimented organisms were rubbed up with saline and re-centrifuged. The washed sediment was made up in saline and equal amounts were put in all manometer cups. The total volume in each cup was 1·0 ml. Also 0·05 ml. *M* glucose, 0·1 ml. catalase and 0·1 ml. 1/10 alcohol, or equal volumes of saline, were added to each cup. The oxygen uptakes recorded are the increased uptakes above those of saline controls. Filter-paper moistened with *M* KOH was present in the side bulbs of the manometers to absorb CO_2.

Oxygen uptakes are given in c.mm.

Expt.	Time. (minutes).	Alcohol + catalase (1).	Alcohol + glucose (2).	Glucose + catalase (3).	Sum of (1), (2) + (3).	Alcohol + glucose + catalase.
1	125	2	14	21	37	44
2	180	0	13	59	72	103
3	90	4	4	28	36	57
4	90	8	16	36	60	105

Table I shows the extra oxygen uptake due to the presence of alcohol. In the absence of glucose, alcohol is not oxidized. In the absence of catalase there is a slight extra oxygen uptake. This is due to the fact that the suspensions of centrifuged organisms cannot be entirely freed from catalase after being grown on a medium containing it.

TABLE II.—*Production of Acetaldehyde from Secondary Oxidation of Alcohol.*

36·0 ml. of a 24-hr. glucose-catalase-peptone culture of organism "C" were centrifuged at 12,000 r.p.m. for 20 minutes. The sediment was made up in 2·5 ml. saline and 0·15 ml. phosphate buffer pH 7·6, 0·3 ml. *M* glucose, 0·3 ml. catalase were added. One ml. of this suspension was put into each of two manometer cups; 0·1 ml. 1/10 alcohol was added to one cup and 0·1 ml. saline to the other. One ml. of the suspension was taken for a blank estimation of volatile aldehydes.

1. *Extra oxygen uptake due to alcohol.*

Time (minutes).	Glucose + catalase (c.mm.).	Glucose + catalase + alcohol. (c.mm.).	Extra O_2 uptake (c.mm.).
170	111	165	54

2. *Acetaldehyde production from alcohol.*

The total contents of each Warburg manometer were taken for the estimation.

Original suspension	0·0 mg.
Glucose + catalase	0·04 mg.
Glucose + catalase + alcohol	0·24 ,,
Extra acetaldehyde	0·20 ,,

From the equation $2C_2H_5OH + O_2 \rightarrow 2CH_3CHO + 2H_2O$, 51·4 c.mm. O_2 corresponds to 0·2 mg. acetaldehyde. The observed oxygen uptake was 54·0 c.mm.

Volatile aldehyde was estimated by distillation into sulphite and titration of the bound sulphite with dilute iodine. The method and apparatus designed by Friedemann, Cotonio and Shaffer (1927) for the estimation of lactic acid was used. In the case of lactic acid, this is first oxidized to acetaldehyde and this is then distilled into sulphite. The oxidation is unnecessary when a direct estimation of acetaldehyde is carried out, and in place of dilute $KMnO_4$, which is used as the oxidizing agent for lactic acid, water is dropped into the distilling flask at a comparable rate.

Keilin and Hartree found that the secondary oxidation of alcohol gave acetaldehyde. Acetaldehyde is not oxidized by the organism "C", so that it should be a detectable end-product. After oxidation of alcohol in the presence of organism "C", glucose and catalase, the suspensions smelt of aldehyde and gave positive Rimini tests for acetaldehyde. One large-scale experiment was carried out in which the extra oxygen uptake due to alcohol was measured and the extra volatile aldehyde produced was estimated.

As shown in Table II these quantities corresponded to the equation:

$$2C_2H_5OH + O_2 \rightarrow 2CH_3CHO + 2H_2O.$$

As Keilin and Hartree found that this oxidation of alcohol was carried out by catalase and H_2O_2, it is reasonable to suppose that H_2O_2 is produced by the oxidation of glucose by the organism "C".

THE TOXICITY OF HYDROGEN PEROXIDE.

It then became necessary to determine whether H_2O_2 destroyed the organism; in fact, whether catalase removed some toxic substance produced from glucose. Experiments were therefore devised to find out if the glucose oxidase was more rapidly destroyed in the presence of glucose than in its absence and whether the addition of catalase had any effect. Suspensions of organism "C" centrifuged out of the medium washed once with saline and

TABLE III.—*The Effect of Glucose on the Activity of the Glucose Oxidase.*

64 ml. of a 24-hr. glucose-blood-peptone culture of organism "C" were centrifuged; the sediment was washed once with saline and suspended in a small volume of saline in the usual way. 1·5 ml. of this suspension was put into Thunberg tubes and 0·1 ml. *M* glucose, 0·1 ml. catalase were put into the hollow stoppers as required. 0·1 ml. *M*/2 phosphate buffer pH 7·6 was added to all tubes. All tubes were evacuated and filled with washed N_2 or H_2 five times. Those tubes which were to be incubated anaërobically were finally filled with gas, the stoppers were closed and the solutions in the stoppers mixed with the organisms. Those tubes which were to be incubated aërobically were finally opened to the air and the solutions in the stoppers were then mixed with the suspension of organisms. All tubes were incubated at 30° C. for 1 hour and then glucose and catalase were added where necessary; thus the final concentration of glucose and catalase was the same in all tubes. 1·0 ml. from each tube was pipetted into Warburg manometer cups and the O_2 uptake was measured in the usual way. O_2 uptakes are given in c.mm. per hour. The O_2 uptake of unincubated organisms was also determined.

Expt.	Anaërobic.	Aërobic.			Unincubated control.
	Glucose.	Saline.	Glucose.	Glucose + catalase.	Glucose + catalase.
1	..	50	6	46	47
2	..	100	33	133	118
3	12	30	0	..	35
4	23	..	5	33	..
5	32	..	11	30	..
6	67	..	47	67	..
7	..	..	3	26	..
8	..	18	9	26	..

then suspended in saline were left at 30° C. for an hour either with or without the addition of either glucose or glucose and catalase. Suspensions of organism " C " were also incubated anaërobically in the presence of glucose to see whether glucose had any effect when no production of H_2O_2 was possible. After incubation with glucose in air, organism " C " had a much smaller respiration than after incubation with saline or with glucose and catalase. Anaërobically, in some cases glucose had a slight destructive action. This was not affected by the addition of catalase, and was never as great as the aërobic destruction.

The experimental results show that glucose, in air, destroys the glucose oxidase, and that catalase prevents this destruction. It seems probable that H_2O_2 is the destructive agent. Catalase might therefore serve two purposes in enabling the filter-passer to grow in otherwise unsuitable media. It can remove H_2O_2 which is produced by the oxidation of glucose and destroys the glucose oxidase, and it can catalyse the oxidation of alcohol present in the medium.

THE PRODUCTS OF GLUCOSE OXIDATION.

In the earlier paper on the oxidation of glucose by the organism " C " it was pointed out that glucose was not oxidized to completion, 3 atoms of oxygen being taken up for every molecule of glucose that disappeared. It seemed possible that alcohol was produced from glucose and that the catalase–H_2O_2 system oxidized it to acetaldehyde. The volatile sulphite-binding compounds were therefore estimated after the oxidation of glucose by the organism either in the presence or absence of sulphite as a fixative. It was found that there was an increase in volatile sulphite-binding compounds after glucose oxidation by the organism " C ". The suspensions gave positive Rimini tests, and in some cases a smell of acetaldehyde could be detected. It seems most probable that acetaldehyde is the volatile compound produced from glucose, but since it has not been isolated it seems better to refer, in the table of results, to the iodine titration obtained, which is directly proportional to the amount of aldehyde produced, than to give the results in terms of acetaldehyde. The presence of sulphite made little difference to the amount of aldehyde produced, indicating that the compound is not further oxidized by the organism, even in the absence of a fixative. There was also an increase in non-volatile sulphite-binding compounds after glucose oxidation, and this increase, also, was not affected by the presence of sulphite. The nature of the non-volatile compound or compounds was not further investigated.

The question now arises whether catalase acts in the medium, whether it is adsorbed by the organism, or whether it is incorporated in the structure of the organism. Owing to the size of the catalase molecule it would probably have to be broken down before it could be taken up by the organism and catalase resynthesized in the organism itself. If organism " C " is grown on a medium to which peptic-digested blood has been added a good growth appears. Organisms centrifuged from this medium are heavily contaminated with hæmin or hæmin breakdown products even after two or three washings with saline. But such washed organisms often have no respiration in the absence of added hæmin or catalase. After the addition of the usual amounts of hæmin or catalase to the suspension of organism, glucose is readily oxidized.

This seems to indicate that the filter-passer " C " does not synthesize catalase from hæmin.

TABLE IV.—*Production of Volatile Sulphite-binding Compounds from Glucose.*

64 ml. of 24-hour glucose-catalase-peptone cultures were centrifuged; the organisms were washed on the centrifuge and then suspended in about 5·0 ml. saline; 0·4 ml. *M* glucose, 0·4 ml. catalase and in some cases Na_2SO_3 to a final concentration of 0·5 p.c. were added. Three ml. of this suspension were pipetted into a large Warburg manometer and this was shaken for 3 hours at 30° C. A blank estimation of volatile sulphite-binding compounds was carried out on 1·0 ml. of the remaining fluid. After 3 hours the Warburg manometer was taken off the bath and the cup cooled in ice before being disconnected. If sulphite had been added to the suspension of organisms the distillation was carried out in the presence of excess $NaHCO_3$.

The results represent the increase in sulphite-binding compounds shown by 1·0 ml. of the suspension of organism after glucose oxidation.

Expt.	Oxidation without sulphite. Sulphite-binding compounds (ml. of 0·0046 *N* iodine).	Oxidation in the presence of sulphite. Sulphite-binding compounds (ml. of 0·0046 *N* iodine).
1	..	0.6
2	0·45	0·45
3	0·85	..
4	0·65	0·80

1·0 ml. 0·0046 *N* $I_2 \equiv 0·101$ mg. CH_3CHO.

TABLE V.—*Removal of Hæmin and Catalase by Washing.*

64 ml. of 24-hr. cultures of organism " C " used.

1. *Removal of Hæmin.*—Organism " C " grown on pepton, digested-blood and glucose, centrifuged and washed once.

Oxygen uptakes given in c.mm. per hour.

Expt.	Saline.	Glucose.	Glucose + catalase.
1	7	10	36
2	10	14	37
3	8	9	36
4	0	0	28
5	..	0	24

2. *Removal of Catalase.*—Organism " C " grown on peptone, catalase and glucose.

Expt.	Saline.	Glucose.	Glucose + catalase.	Number of washes.
1	0	96	98	0
	0	13	46	2
	0	6	24	3
2	2	8	20	3
3	0	17	40	1

If the organism is grown on a medium containing excess catalase the unwashed organisms obtained by centrifugation show no greater respiration when catalase is added. But after washing the organisms two or three times

with saline the addition of catalase increases the respiration by 2–300 p.c. After growth on a catalase-containing medium it is never possible to wash the organisms free from catalase. They always show some power to oxidize glucose even in the absence of added catalase.

This may mean that catalase is adsorbed on to the surface of the organism or that it is incorporated into it. The amount retained is, however, insufficient to enable the organism to respire maximally, and the addition of further catalase causes a very great increase.

DISCUSSION.

There seem several points of interest in this system. There is, first of all, a direct relationship between catalase and growth. Anaërobically the organism "C" will grow well on Difco proteose peptone without catalase and does not utilize glucose. Aërobically it will grow feebly without catalase although the Difco proteose peptone does not contain any hæmin. If we consider that the main function of calalase is to remove peroxide, then we might expect slight growth on a complex medium such as peptone, which almost certainly contains substances capable of being directly oxidized by H_2O_2.

The second point of interest seems to be the relationship between the activity of the glucose oxidase and growth. This enzyme seems to be the only active oxidase in the organism, and just as catalase is necessary for growth, so catalase is necessary to preserve the activity of the enzyme.

A third point of interest is the lack of synthetic power possessed by the organism. The hæmophilic protozoa are unable to synthesize protohæmin, but when this is added to their medium they build cytochrome from it. But organism "C" does not seem to stock itself up with catalase or to synthesize catalase from hæmin, since one saline wash is sufficient to remove all catalase-active compounds from the organisms after they have been grown on a medium containing hæmin. After growth on a catalase-containing medium all traces of catalase cannot be removed by washing, but the amount retained by the organism is so small that it seems probable that it is retained mechanically, rather than that it is the product of a synthesis carried out by the organism itself.

SUMMARY.

In the presence of very small amounts of catalase the filter-passing organism "C" from sewage oxidizes glucose, whereas in the absence of catalase no oxidation takes place. Hydrogen peroxide is probably produced, as a secondary oxidation of alcohol can be observed.

In the presence of glucose the glucose oxidase is destroyed in air. The addition of catalase stops this destruction. One function of catalase therefore seems to be the removal of H_2O_2, which destroys the glucose oxidase.

After glucose oxidation in the presence of catalase there is an increase in volatile sulphite-binding compounds, and the suspensions show a positive Rimini reaction for acetaldehyde. The intermediate steps of glucose oxidation are unknown, but it seems possible that alcohol is produced from glucose, and

that this is further oxidized by the catalase H_2O_2 system to acetaldehyde. This is not further oxidized by the organism.

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INFLUENZA VIRUS ON THE DEVELOPING EGG: VII. THE ANTIBODIES OF EXPERIMENTAL AND HUMAN SERA.

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THERE are three established methods by which antibodies against a given strain of influenza virus can be titrated. These are (1) by the intranasal administration to mice of graded mixtures of serum and mouse-adapted virus (Laidlaw, *et al.*, 1935), (2) by the titration of serum-egg-adapted virus mixtures on the chorio-allantois (Burnet, 1936*b*), and (3) by the complement-fixation test (Smith, 1936; Fairbrother and Hoyle, 1937; Lush and Burnet, 1937). We have already reported (Burnet, Keogh and Lush, 1937) that the results obtained by these three methods do not necessarily correspond. The present communication represents a more detailed study of the problems raised by this lack of correspondence. In view of the great importance which has been attached to antibody titrations of human sera from the point of view of the epidemiology of influenza, it is clearly important that the question of the

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significance of the different types (or manifestations) of antibody should be gone into. In particular the existence of antibody against swine influenza virus in most adult human sera has been interpreted in different fashions by Laidlaw and Shope on the one hand, and Francis on the other. The present work has been done largely in the hope of throwing light on the significance of these swine type antibodies. With the recognition of the sharp antigenic difference between Old World and New World human strains (Burnet, 1937*b*), it became necessary to widen the field of inquiry and use all three antigenic types of virus.

The present paper represents the consolidated experience of two years' work on the virus antibody content of various types of sera. The bulk of the work has been done with the egg membrane technique, but a large number of titrations have been carried out by mouse inoculation methods, and by complement-fixation. In order to present the results as concisely as possible, we have divided the paper into three sections. The first section (A) presents the detailed results of titrating eight sera, chosen to represent each of the main types of serum which had been encountered in the preliminary studies. The sera were quantitatively titrated by each method against each antigenic type of virus. In the second section (B) the antibody content of human sera from various age-groups, as tested by egg membrane titrations against each antigenic type, is described, particular attention being paid to sera from children and adolescents. Finally, in section C we give the results obtained from antibody absorption experiments, which were designed to analyse more closely the characteristic differences between juvenile and adult human sera.

A. COMPARISON OF EIGHT REPRESENTATIVE EXPERIMENTAL AND HUMAN SERA.

The sera of group A were as follows :

1–3. Three ferret immune sera from animals convalescent after infection by " Melbourne " (F. 51), " W. S." (F. 63) and " Swine " (F. 55) strains of virus.

4. The immune horse-serum of Laidlaw *et al.* (1935) (I.H. 2) produced by hyperimmunization of a horse with ferret material infected with strain " W.S."

5. A relatively high titre human serum (K.) from a subject who had a severe attack of presumptive influenza in 1935.

6, 7. Two samples from an adult who showed a well-marked antibody response to the intranasal inoculation of living virus, egg-adapted " Melbourne " strain. The samples R. 1 and R. 2 were taken before and 1 month after inoculation.

8. Serum from a child, aged 4 years, which was known to have a relatively high titre of antibody against " Melbourne ".

Titration methods.—Egg membrane titrations were made as described in previous papers (Burnet, 1936*a* and *b*, 1937*b*), egg-adapted virus of the strains " Melbourne ", " W.S." and " Swine " being used. All sera were heated to 55° C. for 30 minutes soon after they had been separated from the clot, and stored in ampoules or sealed tubes in the refrigerator until used. The only

exception to this rule was the serum I.H. 2, which was received from Dr. C. H. Andrewes about two years previously in liquid form. This was kept in the refrigerator and used without heating. Mixtures were kept in the refrigerator for about 1 hour before being inoculated on fertile eggs at the twelfth day of incubation. After inoculation, eggs were incubated at 36° C. and opened at 40–48 hours in the case of "Melbourne" and "W.S." strains, and at 66–72 hours for the less active "Swine" strain. The results with the latter strain are less satisfactory than with the two human strains, and a wider margin of error must be allowed in the results. The titre of the serum is expressed primarily as the percentage to which the control count of specific foci is reduced when undiluted serum is added to an equal volume of virus. This percentage is calculated from the experimental results on the basis of two rules which have been fully discussed by Burnet, Keogh and Lush (1937). These are: (1) the "percentage law" of Andrewes and Elford that a given antiserum concentration will reduce the number of foci to a constant percentage irrespective of the absolute concentration of virus present, and (2) the "C.P. = constant" rule that serum concentration × percentage of "surviving" foci will give a constant value. As an example, if a serum diluted 1 : 100 mixed with stock virus reduces the count of foci to 0·1 p.c. of the control, it is assumed that undiluted serum would reduce the count to 0·001 p.c. In many circumstances it is preferable to give a positive figure for the antibody content of a serum, and for this the reciprocal of the percentage reduction is used. In the instance cited, the serum would be given an antibody value of 1000.

Mouse titrations were made by the method of Laidlaw *et al.* (1935). Serial 5-fold dilutions of serum over a suitable range were mixed with equal volumes of undiluted stock virus prepared by grinding finely one consolidated mouse lung with 5 c.c. broth and centrifuging in an angle centrifuge for 10 minutes. The supernatant fluid, which was only slightly opalescent, constituted the stock virus. Three mice were inoculated with each mixture, 0·04 c.c. being given intranasally under anæsthesia. Young mice of 15–20 gm. weight were used. Surviving mice were killed on the sixth or seventh days and the extent of consolidation recorded. The end-point was taken as 40–50 p.c. consolidation of the lungs, and the strength of the serum expressed as 10 times that dilution which, mixed with stock virus, produced an average lesion of this degree. The degree of consolidation was very uniform in mice inoculated with the same mixtures. Where none of the experimental series showed the chosen end-point, a value was interpolated from consideration of the degrees of consolidation recorded.

The complement-fixation tests were carried out as described by Lush and Burnet (1937), and the figure given is the highest final serum dilution which, with optimal antigen concentration, fixed 3 M.H.D. of complement.

The question of using a standard serum and expressing the antibody content of other sera in terms of this has been considered, but, as will be seen when the results are discussed, it is very difficult to decide on what type of serum to use. Actually we have generally used the convalescent ferret sera F. 51, F. 63 and F. 55 as standards for egg membrane and mouse titrations of the corresponding virus strains, and the human serum K as a standard positive serum for complement-fixation tests. In the present work all sera have been

tested in each fashion in a single experiment, and as relative values only are required, the question of an absolute standard does not arise. For convenience, the antibody values are also expressed (Table II) in terms of our sample of serum I.H. 2. This has been preserved as liquid in the refrigerator for two years, and may have deteriorated considerably, but it is the only experimental serum we possess with a wide range of activity against all three virus strains.

TABLE I.—*Titration of Representative Sera by Different Methods.*

Serum.	Egg membrane titrations.			Mouse titrations.			Complement-fixation.	
	Melbourne. %	W.S. %	Swine. %	Melbourne.	W.S.	Swine.	Melbourne.	Swine.
F. 51. Convalescent ferret "Melbourne"	0·001	1·0	22	4000	<10	<1*	100	50
F. 63. „ ferret "W.S."	1·1	0·004	2·4	<20	700	..	100	100
F. 55. „ „ "Swine"	13	9·5	0·0012	2*	..	1000	12·5	50
K. Adult human . . .	0·39	0·2	0·006	220	40	250	100	100
R. 1. „ „ before immunization	5·2	1·0	0·0012	80	20	1000	12·5	12·5
R. 2. Adult human. After immunization with "Melbourne" egg virus	0·15	0·63	0·0012	80	25	1200	12·5	12·5
Ga. Child aged 4 years . .	0·078	10	12	180	<10	<1*	0	0
I.H. 2. Standard horse-serum .	0·077	0·025	0·02	400	140	50	12·5	12·5

* Values derived from results of inoculating undiluted serum + virus diluted 1 : 10.

TABLE II.—*Comparative Activities of the Same Sera in Terms of those of Serum I.H. 2.*

Serum.	Egg membrane titrations.			Mouse titrations.		
	Melbourne.	W.S.	Swine.	Melbourne.	W.S.	Swine.
F. 51 . .	7700	2·5	0·09	1000	<7	<2
F. 63 . .	7	625	0·8	<5	500	..
F. 55 . .	0·5	0·25	1600	0·5	..	2000
K . . .	20	12·5	320	55	28	500
R. 1 . .	1·5	2·5	1600	20	14	2000
R. 2 . .	52	4	1600	20	18	2400
Ga . . .	100	0·25	0·16	45	<7	<2
I.H. 2 . .	100	100	100	100	100	100

An examination of Tables I and II will show a number of points of interest.

1. Neutralization tests with convalescent ferret sera are highly specific, but complement-fixation tests are much less so. These points have been previously discussed (Burnet, 1937*b* ; Lush and Burnet, 1937).

2. Adult human sera show a very high content of antibody capable of neutralizing swine type virus by either method.

3. Normal adult human sera show fairly comparable amounts of antibody against "Melbourne" and "W.S." strains.

4. An adult inoculated intranasally with egg passage " Melbourne " virus shows a sharp rise in antibody detectable by egg membrane titration against the homologous strains, but no significant changes in the antibody determined by other methods of titration.

5. Serum from a young child contains relatively large amounts of neutralizing antibody against " Melbourne " strain, but only insignificant traces against the other two strains.

6. The horse-serum I.H. 2 shows a wide range of activity against the three virus types.

Each of the points 1 to 5 has been found generally applicable to other sera of the corresponding types.

B. THE ACTIVITY OF HUMAN SERA DURING AN INTER-EPIDEMIC PERIOD AGAINST THE THREE ANTIGENIC TYPES OF INFLUENZA VIRUS.

It seemed to us that more information of value would be gained from a detailed examination of small groups of sera from individuals in each main age-group, particularly the adolescent period (10–17), than by accumulation of data from random adult sera.

In Figs. 1 and 2 are represented graphically the antibody titres determined by egg membrane titration of representative sera from the following age-groups :

(1) At birth (umbilical cord blood).
(2) Infants from 3 months to 1 year.
(3) Young children (3–10 years).
(4) Adolescents (11–17 years).
(5) Adults.

Each serum was titrated against each antigenic type, using the egg-adapted strains " Melbourne ", " W.S." and " Swine ". The activity of each serum is expressed logarithmically, using an inverted scale of the percentage to which the count of specific foci is reduced by undiluted serum.

The sera in groups 1, 2 and 4 are random samples. The 4 sera from young children (group 3) were chosen as those containing the highest antibody titre from a large number which have been tested against " Melbourne " strain on the egg membrane. A majority of young children in Melbourne show no antibody, or much smaller amounts than these do (*see* Fig. 3).

The five adult sera were chosen to provide a sample of each of the main types of serological behaviour which we have encountered in a much larger series of tests.

Taking first the incidence of swine type antibodies, we see the characteristic difference between the activity of sera from young children and from adults, which has been previously described from England and America. Like all other antibodies which have been studied in this respect, influenza virus antibodies of each type pass into the fœtal circulation, but are rapidly lost during the first months of independent life. During childhood those children who have presumably been infected show the appearance of highly specific antibody. Over the transition period 10–16 there are the inevitable irregularities, but there is a clear indication of a gradual increase in swine type antibody over

this period. It is not at all the type of finding one would expect if the swine type antibody were a relic of an infection by " pandemic " influenza, occurring at a very early age, some time between 1920 and 1925.

The serum from the child F. 12, which showed unusually high " Swine " antibody for this age (12 years), was also tested in mice. It was of similarly

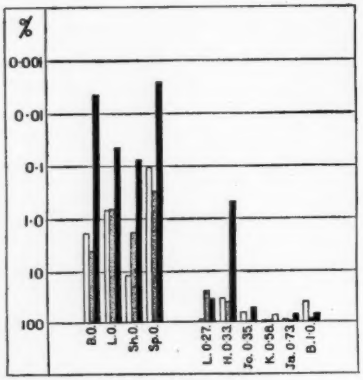

FIG 1.

FIGS. 1 and 2.—Antibody titres of human sera from individuals of various ages as titrated by egg membrane methods against the strains " W.S.", " Melbourne " and " Swine " of influenza virus. The titre is shown logarithmically in terms of the reduction in count of membrane foci using an inverted percentage scale. The vertical columns indicate respectively titres against " W.S." white, " Melbourne " stippled, and " Swine " black for each serum. The sera are arranged in order of age in five groups. The age in years and a distinguishing initial are shown beneath each set of titrations.

FIG. 1.—Sera from umbilical cord blood of newborn infants, and from infants from 3 months to 1 year.

FIG. 2.—Sera from a selected group of young children showing high antibody titres, an unselected series of adolescents from 10 to 17 years, and a small group of adults selected to show different combinations of antibody titres.

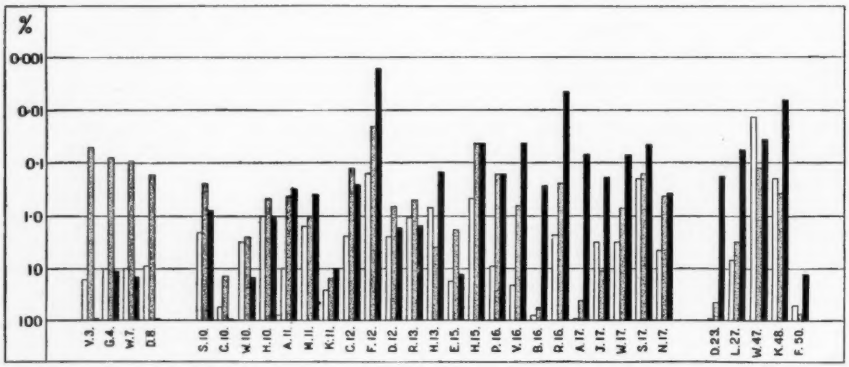

FIG. 2.

high titre, giving a value of 300 according to the convention used in Table I (600 in Table II). As a curiosity it may be noted that the adult subject F., who shows practically no antibody to any type, suffered a typical attack in the 1918 pandemic and has never since had recognizable influenza.

The relative activity against " Melbourne " and " W.S." strains also shows features of interest. In young children who show any antibody it is predominantly against the " Melbourne " strain. In adolescents and adults there is a much closer correlation between the two, " Melbourne " titres being usually

only a little higher than " W.S.". Several subjects, including one 13-year-old, show a significantly higher titre against " W.S." than against " Melbourne ". The obvious interpretation is that the antibody found in the sera of a proportion of young children was induced by infection in 1935 with the " Melbourne " strain then widely prevalent. In previous years epidemics due to various strains, some of the " W.S." antigenic type, must have occurred in Australia, leaving certain individuals with antibody predominantly against this type.

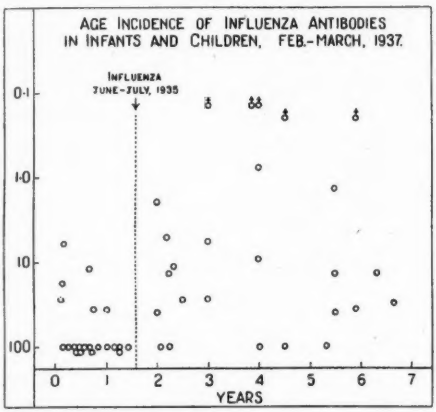

FIG. 3.—Antibody titres against " Melbourne " strain on the egg membrane of sera from infants and children, taken in February and March, 1937, approximately 20 months after an influenza epidemic due to " Melbourne " type virus. Ordinates antibody titre; abscissæ, ages of subjects in years.

In Fig. 3 are shown the results of titrating a large series of sera from infants and young children, taken in February and March, 1937, against " Melbourne " strain. Unfortunately at this period we had not yet adapted " W.S." or " Swine " to egg titrations, so that the information is less complete than it might be. The diagram shows very clearly, however, the relationship between antibody and probable exposure to infection. Of children born since the last appearance of influenza in Melbourne and more than 3 months old, 13 had no trace of antibody, 3 had traces (10–100 p.c.), none any appreciable amount. Of those born before this epidemic, 5 had no trace, 10 traces, 5 medium amounts, and 6 large amounts of antibody.

C. ANTIBODY-ABSORPTION EXPERIMENTS.

We have previously shown that a certain proportion of antibody can be absorbed from convalescent ferret serum by treatment with infected tissues (Burnet, Keogh and Lush, 1937). In general, the antibody demonstrable on the egg membrane is much more readily absorbed than that demonstrable in mouse tests. With some sera it was impossible to demonstrate any absorption of " mouse antibody "; others showed absorption of both types if a large enough absorbing dose were used. This work was done using only the " Melbourne " strain virus and homologous antisera.

In the hope that absorption experiments would throw light on the nature of swine type antibodies, both experimental ferret sera and high titre adult

human sera were absorbed with swine virus (infected mouse lungs) and the absorbed sera titrated. The technique was similar to that previously used. A suitable dilution of serum was prepared, and 2 c.c. added to a ground-up paste of two consolidated lungs. The emulsion was allowed to stand overnight in the refrigerator, centrifuged, and the supernatant fluid heated to 55° C. for 30 minutes. A precipitate usually formed on heating, and this was removed by a final spinning. The supernatant fluid represented the absorbed serum. Many tests have shown that this treatment has no damaging effect on unabsorbed antibodies, and appears actually to protect the antibody against the slight inactivation which occurs when dilute serum is similarly heated. The controls therefore were unabsorbed, and unheated serum diluted to 5/4 the degree of the absorbed serum to allow for the volume of tissue added.

The results obtained are shown in Table III, the degree of absorption being expressed as percentage reduction in antibody activity.

TABLE III.—*Antibody Absorption Experiments.*

Serum.	Absorbed with—	Percentage reduction in antibody activity as tested against—			
		"Swine" (mouse) %	"Swine" (egg) %	"Melbourne" (egg) %	"W.S." (egg) %
F. 55. (1 : 50) Ferret ("Swine") .	"Swine" (mouse) .	90±	100	..	..
	„ (egg) .	75±	90·5	..	..
	"Melbourne" (mouse) .	..	*Nil*	..	..
F. 56. (1 : 50) „ „ .	"Swine" (mouse) .	*Nil*	100	..	..
K. (1 : 40) Adult human . .	„ „ .	100	100	..	..
K. (1 : 5) „ „ . .	„ „ .	99±	99·65	81	98·9
W. (1 : 2) „ „ . .	„ „ .	..	84	91·5	99·3
W. (1 : 5) „ „ . .	„ „ .	..	99·95	98	99·9
V. (1 : 5) Child aged 3 years .	„ „ .	..	..	*Nil*	..

It will be seen that the ferret sera behave in the same way as homologous ferret sera did with the "Melbourne" strain. F. 56 shows complete dissociation of "egg" and "mouse" antibody, while F. 55 shows considerable reduction by both methods. A heterologous strain "Melbourne" has no absorbing power at all.

The human sera behave quite differently, "Swine" virus being capable of absorbing all types of antibody, *including heterologous activity*, with almost equal readiness. The two human sera tested, K and W, show some differences in behaviour, but there is no doubt at all that very considerable amounts of "Melbourne" and "W.S." antibody are removed. It is probably significant that more activity against "W.S." is removed in each case than against "Melbourne", which was presumably responsible for the clinical infection which both these subjects suffered in 1935.

From the general resemblance between children's sera and those of convalescent ferrets, it would be expected that absorption with "Swine" virus would have no effect in reducing the titre of the specific "Melbourne" antibody. This is found experimentally. Serum V. 3, diluted 1 : 5, was absorbed

according to the usual technique with "Swine" mouse lung virus. No absorption whatever was observed. As is usual in such experiments, the absorbed serum seemed to be slightly, but not significantly, more active than the unabsorbed.

DISCUSSION.

Before discussing the significance of swine influenza antibodies in human sera, we may consider some of the more general points raised by the results shown in Tables I and II.

The changes in the serum of subject R. after intranasal inoculation of living egg-adapted "Melbourne" virus are of particular interest. During the autumn of 1937 about 200 individuals were inoculated in this way with a view to determining whether any protection against clinical influenza could be so afforded. No reports of symptoms which could be ascribed to the inoculations were received, so that it can be taken that the egg-adapted virus has lost its pathogenicity for human beings in the same way as it has for ferrets (Burnet, 1937*a*). As no influenza appeared in Melbourne during the winter, no evidence of protection could be obtained. Eighteen individuals so treated were tested for antibody before and after the inoculation. By the egg membrane titration method about half showed no detectable change, 6 showed slight rises in antibody activity from 2 to 5 times that originally present, and 2, including R., showed an increase to more than 10-fold. Both these sera show the same clear-cut rise in "egg" antibody, and no significant change in the antibody demonstrable by mouse inoculation.

It has been shown previously (Burnet, Keogh and Lush, 1937) that immune ferret sera can be absorbed almost free of antibody demonstrable on the egg membrane, but show no change in the amount of antibody demonstrable in mice. The inference was drawn that only a small proportion of more highly avid antibody molecules were concerned in the egg membrane neutralizations. The results with R. 1 and R. 2 provide confirmation of this point of view from the opposite direction, the active addition of antibody to the serum following immunization causing a large increase in the egg membrane titre, but not influencing the other activities. It is hard to avoid the interpretation that a small amount of "high-grade" specific antibody has been added to the blood, which already contains a much larger number of molecules of "low-grade" antibody of less specific activity.

In this connection we may refer to differences in the ratio between the amount of antibody detectable by egg tests and that found by tests on mice (E/M ratio). Insufficient work has been done with "W.S." in mice to allow conclusions, but with the "Melbourne" strain it has been found regularly that the E/M ratio was much higher for convalescent ferret sera than for normal adult sera or the standard serum I.H. 2. In Table II it will be seen that, taking the E/M ratio for I.H. 2 as unity, that for ferret 51 is 7·7, and for the 2 normal adult sera, 0·36 and 0·075. The last value is an unusually low one. Similarly with "W.S." the E/M ratio for ferret 63 is 1·25, for the normal adult sera 0·45 and 0·18.

This would naturally be interpreted as indicating that convalescent ferret serum contained a much higher proportion of high-grade specific antibody

than normal adult human serum. The child's serum Ga. is intermediate in character, showing an E/M ratio for " Melbourne " of 2·2. This also contains a relatively high proportion of high-grade antibody.

When we examine the E/M ratio for " Swine " virus, we find a significant difference. The ferret serum 55 shows a ratio of 0·8, the adult human sera 0·64 and 0·8, *i. e.* convalescent ferret, adult human and hyperimmunized horse-sera, all show reasonably similar ratios between egg membrane and mouse titrations. In other words, when antibody has any significant action at all on swine type virus, it behaves as if it were of high-grade type.

At one stage we were inclined to regard the antibody persisting in adult human sera during interepidemic periods as of low-grade type more or less equivalent to that remaining in ferret immune sera after absorption with homologous virus. The results of absorption of adult sera with " Swine " virus shown in Table III make it clear that such is not the case. The antibody present is all capable of being absorbed by " Swine " virus, and must be regarded as just as " avid " as the specific antibody of ferret or children's sera, but with a more generalized range of combining power. The absorption experiments at once dispose of any contention that the power to neutralize " Swine " virus is due to antibody produced in response to an infection (during the period 1918–1925, or at any other time) by virus of this specific antigenic type. This is not to say that human beings have never been infected with this type of virus, but merely that the presence of neutralizing antibody supplies no evidence which is relevant to the matter. The evidence put forward by Laidlaw (1935) and by Shope (1936) in favour of the view that such swine type antibodies are a result of infection by the virus responsible for the pandemic of 1918–19 is well known. Their view that " hog flu " first appeared in the American middle West immediately after the pandemic, and was in all probability directly derived from the human infection, appears to be generally accepted.

This in itself, however, does not seem sufficient to justify regarding the human antibodies as having been produced by pandemic type virus. In view of what is known of the rate at which specific antibody titres fall after the antigenic stimulus ceases, it would be extraordinarily unlikely that a child of 12, born in 1925 and infected within its first year or two, should still retain a high content of specific antibody. If any alternative theory is available, it would obviously be preferable.

We consider that the experimental evidence provided in this paper justifies the following description of the development of influenza virus antibodies in an urban population of human beings. The infant at birth possesses the same antibodies as its mother, but loses these within a few months, and shows no antibody against any type of influenza until it is infected by the virus. The antibody produced after infection in childhood is highly specific, showing the same differentiation between the three primary antigenic types of virus as is shown with convalescent ferret sera. During the period of adolescence (10–16 years) there is a gradual appearance of a " generalized " antibody active against all three types, but particularly so against the most readily inactivated type " Swine ". Following infection in adult life, there is a temporary appearance of specific homologous antibody, and if the infection reaches a

clinical level, an increase in the amount of generalized antibody also occurs (Smith and Stuart Harris, 1936). In the absence of fresh clinical or subclinical infection there is a steady diminution of the activity of both specific and generalized antibody, but the activity of the latter against swine antibody remains easily detectable in most subjects throughout adult life.

We may consider the evidence for this hypothesis of the nature of the swine type antibodies in human beings from two points of view, concentrating first on the properties of the antibodies, and second on the nature of the virus concerned. Considering the problem first from the side of the serum, we find that although ferrets, even after hyperimmunization, show very little development of heterologous activity against swine type virus, the horse I.H. 2, also subject to hyperimmunization, shows a considerable degree of such activity. It is well known that different species vary in the specificity of the antibody they produce against a given micro-organism. Topley and Wilson (1936), for instance, mention that human antibodies produced as a result of Salmonella infections are much less specific in their O agglutinins than the selected rabbit sera used for systematic studies of the group. It is reasonable to expect therefore that adult human sera may show a much lower degree of specificity than ferret sera against the influenza viruses. Direct evidence that such is the case may be found from the literature. In 1935 a large number of human sera were examined both by the Hampstead group of workers and by the Rockefeller Institute workers. Despite the fact that they used respectively the strains " W.S." and " P.R. 8 ", now known to be sharply distinct immunologically, there was a general agreement that sera of high titre against one strain were also of high titre against the other.

In our own direct comparative studies of adult sera on this point, there is a general correlation between the titres against the two human types. Careful studies made by Smith and Stuart-Harris (1936) on the serum of a laboratory worker infected with the strain " W.S." showed that along with the rise in titre against " W.S." there was a well-marked increase in the power of the serum to inactivate " Swine " virus.

Finally, our antibody-absorption experiments with adult human sera show conclusively that the same antibody molecules which inactivate human strains of influenza virus are capable of union with swine type virus. The more specific antibody produced after infection in children by strain " Melbourne ", like that produced in ferrets experimentally infected, is not capable of such union.

Turning to consider the question from the side of the virus concerned, we find indications that swine virus differs from the human types in ways which might make it more readily inactivated than these by low-grade antibody. In the first place, swine virus lacks the high degree of lability which is characteristic of human strains, it infects mice and ferrets without adaptation, and retains a relatively constant virulence on passage. Unlike the human strains, it shows only a very low degree of adaptability to the egg membrane. After more than seventy passages it has developed very little more activity than at the beginning, and has no lethal effect on the embryo. It seems to behave as if it were a fixed variant derived by loss of some component or function from some type more closely resembling the current human strains.

Second, the constancy of the E/M ratio (see above) would indicate that with swine virus what is capable of inactivating the virus in the mouse will also inactivate it on the egg membrane. Actually this is not always correct; absorption experiments with the ferret serum F. 56 ("Swine") show that with swine virus in the form of mouse lung almost complete absorption of antibody active on the egg can occur, with no demonstrable reduction in that responsible for inactivation in the mouse. If we use a high titre adult human serum for this experiment, however, both are absorbed to about equal degree.

Third, complement-fixation results show that the optimal concentration of swine antigen is similar for all types of serum and of a type characteristic of the more avid sera against the "Melbourne" strain antigen.

It is obvious that a complete understanding of the immunological reactions of human sera against influenza viruses can be reached only when there has been an opportunity for many years' immunological study of a given population in the light of the strains known to have been responsible for the successive epidemics. In the absence of such experience, interpretation can be based only on the balance of probabilities. In our view, "Swine" influenza antibodies represent "generalized" residual antibody resulting from repeated infections with human type strains. The alternative view, that they represent antibodies derived from infection with "pandemic" influenza, seems almost untenable. It is worth noting, however, that if any laboratory has available any samples of adult human serum taken prior to 1918 it would be possible to provide direct evidence for one or other of the alternatives. Further, if a community which escaped the 1918 pandemic provided sera which contained a normal content of human type antibodies and no swine type antibody, the pandemic theory would be very greatly strengthened. Neither of these more direct types of evidence has yet been provided.

SUMMARY.

1. Representative human and experimental sera have been tested against the three antigenic types of influenza virus by titration on the egg membrane, by intranasal inoculation of mice, and by complement-fixation.

2. A series of human sera from individuals representative of various age-groups has been studied by egg membrane titration of antibody against all three types of influenza virus.

3. The following are the chief results obtained from such titrations:

(i) Experimental ferret sera are highly specific, show a relatively higher titre in egg membrane tests than in mouse titrations, and show some evidence of specificity in complement-fixation tests.

(ii) The immune horse-serum I.H. 2 has a wide range of activity against all three virus types.

(iii) Sera from young children, when they contain antibody, show highly specific reactions, inactivating only the presumed homologous strain "Melbourne" and being without action in swine type virus.

(iv) Adult human sera regularly contain large amounts of antibody capable of neutralizing swine type virus both in mice and on the egg membrane.

(v) Living egg-adapted " Melbourne " virus can be administered by intranasal spray to human volunteers without causing symptoms. In a proportion of individuals so treated, a relatively high rise in antibody was demonstrable by egg membrane titration, but not by mouse tests.

(vi) Human sera at birth contain antibodies against the three types in amounts similar to those of adult sera. Within a few months all antibodies disappear.

(vii) Significant amounts of " Melbourne " type antibody are found in many young children born before the last local epidemic, not in those born since.

4. Absorption of human serum with swine type virus shows characteristic differences between adult and children's sera :

(i) All types of antibody are removed from adult sera.

(ii) Absorption with swine type virus removes none of the specific " Melbourne " antibody from a high titre serum of a child aged 3.

5. These results are discussed, and it is concluded that the swine type antibody in human serum is not specific, but is a manifestation of " generalized " antibody, developing usually about the time of adolescence as a result of repeated infections with human types of virus.

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A COMPARISON OF THE INTERCHANGE OF THE BODY FLUIDS AFTER INTRAVENOUS INJECTIONS OF CRYSTALLOIDS, GUM ACACIA AND BLOOD-SERUM.

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THE osmotic pressure of the proteins in blood-serum was found by Starling (1895–6) to amount to about 30 mm. Hg. by direct measurement. Moore and Parker (1902) confirmed this, and in addition noted that the colloid osmotic pressure could be made to vary by the addition of diffusible crystalloids, although such crystalloids passed easily and rapidly through the membrane. In a later paper Moore and Roaf (1907) extended these observations. Briefly their conclusions were that the osmotic pressure of the serum proteins fell with the addition of NaCl or any neutral salt, rose considerably after alkalization, and was unaffected by anæsthetics or benzol. Of the colloidal constituents not normally found in the blood-stream the greatest attention has been paid to gum acacia, whose chemical constitution has been investigated by Norman (1929). Bayliss (1919), Gasser Erlanger and Meek (1919), Krogh and Nakazawa (1927) and Dodds and Haines (1934) have shown that, as in the case of blood proteins, the osmotic pressure of gum falls after the addition of NaCl. Bayliss considered that 6 p.c. gum in 0·9 p.c. NaCl was a useful substitute for blood because its osmotic pressure was the same as the blood colloids and its viscosity the same as whole blood. Varying results, however, have been given for the osmotic pressure of 6 p.c. gum in 0·9 p.c. NaCl, thus—8·8 mm. Hg (Dodds and Haines, 1934); 11 mm. Hg (Onozaki and Sanada, 1935); 12 mm. Hg (Knowlton, 1911); 16 mm. Hg (Krogh and Nakazawa, 1927); and 39 mm. Hg (Bayliss, 1915–17). Following Krogh and Harrop's (1921) work on the prevention of capillary dilatation, Drinker (1927) observed the hind feet of a frog after perfusing the anterior tibial artery with 6 p.c. gum in 0·65 p.c. NaCl and 3 p.c. gum in 0·65 p.c. NaCl plus 20 p.c. horse-serum. Although the former had an osmotic pressure of 16 mm. Hg and the latter a pressure of 9 mm. Hg, nevertheless the former was unable to restrain œdema and the latter was. He concluded that serum proteins restrained œdema formation by some other means than by purely their osmotic pressure, and that although gum acacia possessed a colloid osmotic pressure identical with the blood proteins when measured in an osmometer, it did not resemble plasma when placed in a blood capillary. Drinker thought that any reputation held by acacia must depend more upon the company in which it finds itself when placed in the mammalian circulation than upon any significant suitability inherent in the gum.

It was felt that without much more direct and complete evidence the

work of Drinker upon the frog could not be applied, as he has suggested, to the mammalian circulation. In determining the values for the osmotic pressure of gum as well as other colloids, osmometers with membranes impermeable to the colloid were used, yet even under such controlled laboratory conditions wide disparities were obtained by different workers. The differences in the readings have been variously ascribed to variations in the temperature (Krogh and Nakazawa); pH (Gasser, Erlanger and Meek, 1919); salt concentrations (Moore and Roaf, 1907; Bayliss, 1915–17; Dodds and Haines, 1934), and the presence or absence of the sodium or calcium salt (Bayliss, 1915–17; Gasser, Erlanger and Meek, 1919). Even if comparable figures were obtained, it is by no means certain how far the results obtained with osmometers can be applied to the living body. The permeability of the capillary membrane has never been determined because no analyses have been made on the tissue fluid, yet in making laboratory measurements of the osmotic pressure of colloids it has been assumed that the capillary membrane is completely impermeable to the colloid. It was felt that more direct observations should be carried out on the capillary membrane, and the experiments which follow constitute an attempt to study the behaviour of crystalloid solutions, various solutions of gum acacia and blood serum within the mammalian circulation. The experiments were carried out on intact animals and on animals after a controlled hæmorrhage.

METHODS.

Cats under "nembutal" (sodium ethylmethylbutylbarbiturate, Abbots Laboratories, Montreal) anæsthesia were the sole animals employed. The normal blood volume was assumed to be 70 c.c./kg. of body-weight, and the variations in blood volume consequent upon intravenous injections were determined by hæmoglobinometry as described by Robertson (1935).

RESULTS.

The experimental investigations were divided into five main groups. These were the changes which took place in the blood volume (*i. e.* the nature of the interchange of tissue fluid or loss from the vascular system of the injected fluid) after the intravenous injections of—

1. Crystalloid solutions of 0·3, 0·6, 0·9, 2·0, 4·0, 33·0 p.c. NaCl and 50 p.c. glucose.
2. 6 p.c. gum acacia in water and in 0·3, 0·6, 0·9, 2·0 and 4·0 p.c. NaCl.
3. 6·0, 15·0 and 33·0 p.c. gum in water, the gum being given in equivalent amount per kg. of body-weight.
4. Blood serum from cats.
5. 6·0 p.c. gum in 0·9 p.c. NaCl, 15 p.c. gum in water, normal NaCl and blood serum in amounts equal to a controlled hæmorrhage.

In all experiments on the intact animal (groups 1 to 4) the intravenous injection given was 44 c.c./kg. at an average speed of 4 c.c./min./kg. except in the case of 33 p.c. NaCl., 50 p.c. glucose and 33 p.c. gum acacia, which were given at 0·5 c.c./min./kg., and 15 p.c. gum acacia, which was given at

1·0 c.c./min./kg. In the experiment after hæmorrhage (Group 5) the animals were bled at the rate of 2 c.c./min./kg., and the infusion equal in volume to the hæmorrhage was injected at 4 c.c./min./kg. The curves in the figures 1 to 7 represent the changes which take place in the blood volume (calculated as the number of c.c./kg. of body-weight) after the various infusions. The ordinate represents the volume of blood in ml./kg., and the abscissa represents the periods in minutes between the observations (except Fig. 5; periods in

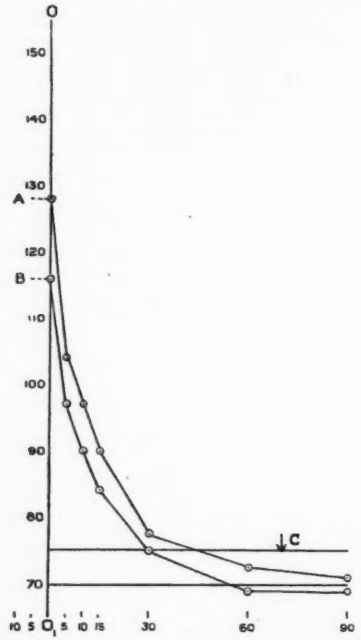

Fig. 1.

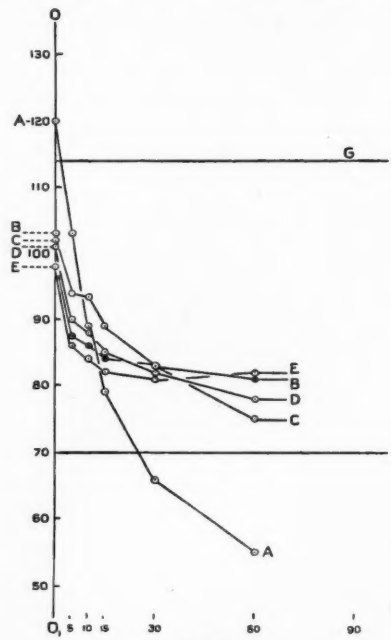

Fig. 2.

Fig. 1.—Intravenous injection of 5 c.c. per kg. of 33 p.c. NaCl and of 50 p.c. glucose. A, 33 p.c. NaCl. B, 50 p.c. glucose. C, Theoretical blood volume per kg. 70 + 5 = 75 c.c. per kg.

Fig. 2.—Intravenous injection of A, 4 p.c. NaCl. B, 2 p.c. NaCl. C, 0·9 p.c. NaCl. D, 0·3 p.c. NaCl. E, 0·6 p.c. NaCl. Normal blood volume = 70 c.c. per kg. G, Theoretical blood volume: 70 + 44 c.c. per kg.

hours). The line 0_1 marks the end of the intravenous injections, and when the first sample of blood was removed for comparison with the normal blood which was taken before the infusion was given.

Experiment 1.—In Fig. 1 are plotted the curves showing the variations in the blood volume after the intravenous injection of strongly hypertonic solutions of NaCl and of glucose. It will be noticed that the increase in volume immediately after the intravenous injection of very hypertonic crystalloids was very marked, and greatly in excess of that caused by the volume of the

injecting fluid. The increase in volume took place during the injection so quickly that the time taken for the transfer of fluid from the tissues was inappreciable. Immediately the injection was stopped the transfer of fluid from the tissues also ceased, and the volume of blood began at once to diminish, ridding itself of the excess fluid, and at the end of an hour the blood volume was normal. The failure to attract fluid from the tissues after the injection had stopped suggested that the force attracting the tissue fluid was no longer

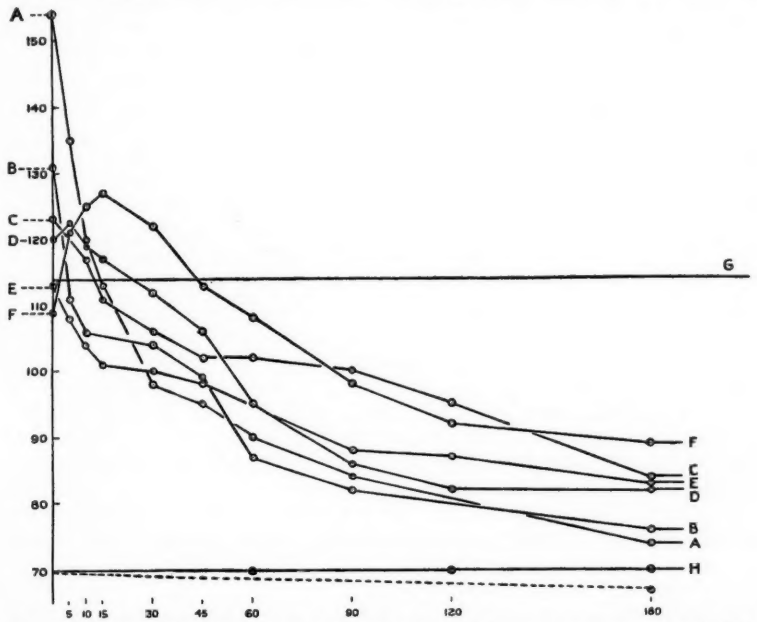

FIG. 3.—Intravenous injection of A, 6 p.c. gum, 4 p.c. NaCl. B, 6 p.c. gum, 2 p.c. NaCl. C, 6 p.c. gum, 0·6 p.c. NaCl. D, 6 p.c. gum, 0·3 p.c. NaCl. E, 6 p.c. gum, 0·9 p.c. NaCl. F, 6 p.c. gum aqueous. G, Theoretical blood volume per kilo after the injection of 44 c.c. per kilo.

present, or that osmotic equilibrium between blood and the tissue had been established, and Leathes (1895–6) has found by actual measurement that this is so.

Experiment 2.—In Fig. 2 are plotted the curves showing the variations in blood volume after the injection of large amounts (equal to 4/7 of the calculated total blood volume) of various percentages of saline. Two fluids in this series were hypertonic, namely the 4 p.c. and 2 p.c. saline. With 4 p.c. NaCl, as would be expected after any hypertonic solution, there is an attraction of fluid during the injection, but as in Fig. 1, immediately the injection is completed fluid rapidly leaves the vascular system and half-an-hour later the blood volume is below normal. With 2 p.c., 0·9 p.c., 0·6 p.c. and 0·3 p.c. NaCl there is little if any difference at all in the interchange between the blood

and body fluids. An hour after the injection of 2·0 p.c., 0·9 p.c., 0·6 p.c. and 0·3 p.c. NaCl the blood volume, though gradually falling, had not returned to normal.

Experiment 3.—A study of Fig. 3 shows that when 6 p.c. gum acacia in water is injected intravenously some fluid leaves the vascular system during the injection. From the fifth to the forty-fifth minute after the injection, fluid is attracted from the tissues to the vascular system; thereafter there is a gradual fall in blood volume, but at the end of 3 hours the volume was still 27 p.c. above normal. When NaCl up to 0·9 p.c. is added, a lowering takes place in the osmotic pressure of the gum acacia and less fluid is attracted from the tissues, but at the end of 3 hours little difference is apparent in the blood volume, which averaged 21 p.c. above normal. When hypertonic NaCl is added to gum acacia a great increase in the osmotic pressure of the infusion takes place. This increase, however, is only active when these solutions are being injected. When the injection is completed fluid rapidly leaves the vascular system, and 3 hours later the mean volume has fallen to 7 p.c. above normal. Thus the gum, when injected with hypertonic NaCl, seems to have lost some property it previously possessed in retaining fluid. While the addition to gum acacia of NaCl in concentration of 0·3 p.c. to 0·9 p.c. brings about a decreasing fall in the osmotic pressure of the gum acacia, the nature of the changes cannot be explained entirely by the presence of the NaCl, for reference to Fig. 2 shows that an injection of 2 p.c. NaCl in no way differs from 0·9 p.c., 0·6 p.c. and 0·3 p.c. NaCl, yet when these solutions are given with 6 p.c. gum the interchanges of fluid differ markedly. Further, with 6 p.c. gum in 4 p.c. NaCl there is produced an increased osmotic pressure greater than can be accounted for by either the gum acacia or the NaCl present.

Experiment 4.—In giving equivalent amounts of gum acacia when injected in solutions of 6·0 p.c., 15 p.c. and 33 p.c. (Fig. 4), the required volume was calculated in the following way on the basis of 44 c.c. per kg. of 6 p.c. gum :

44 c.c. of 6 p.c. gum contains 2·64 gm.
Therefore 100 c.c. of blood contains $2{\cdot}64/114 \times 100 = 2{\cdot}32$ gm.
Let x = c.c. of 15 p.c. gum required.
Then gm. of gum = $15x/100$.
The volume of fluid of the animal in which this circulates = $70x$.

$$\text{The percentage of gum} = 15x/100\ (70x) \times 100/1.$$
$$= 15x/70x.$$
$$\text{and } 15x/70x = 2{\cdot}32.$$
$$15x - 2{\cdot}32x = 162{\cdot}4.$$
$$x = 12{\cdot}8 \text{ c.c.}$$

In the same way it can be calculated that the amount of 33 p.c. gum equivalent to 44 c.c. of 6 p.c. gum or 12·8 c.c. of 15 p.c. gum is 5·3 c.c.

Fig. 4 demonstrates how similar are the curves for 6 p.c. and 15 p.c. gum and how dissimilar is the curve for 33 p.c. gum. There would thus appear to be a quantitative relationship between solutions of gum up to 15 p.c. Dodds and Haines (1934) found that such a relationship existed between the limits of 2 p.c. and 6 p.c. gum when their osmotic pressures were measured in an

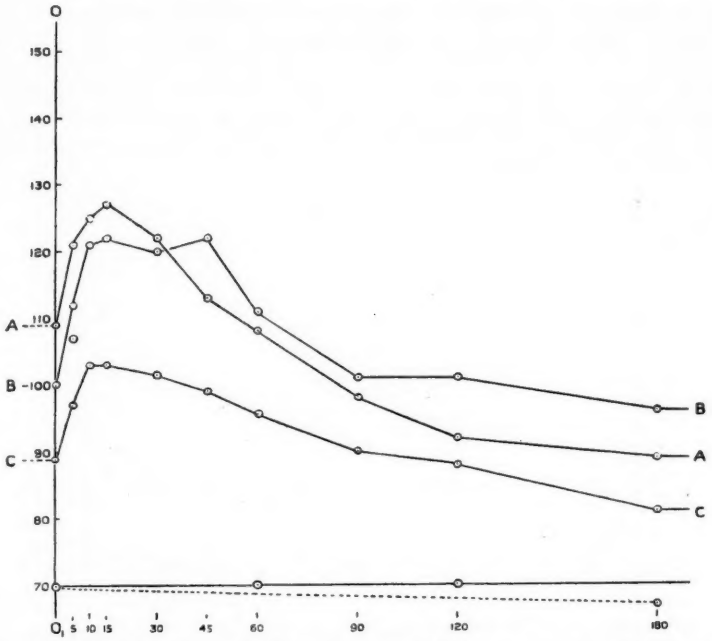

FIG. 4.—Injections of 44 c.c. per kg. of 6 p.c. gum in water (A). 12·8 c.c. per kg. of 15 p.c. gum in water (B). 5·3 c.c. per kg. of 33 p.c. gum in water (C).

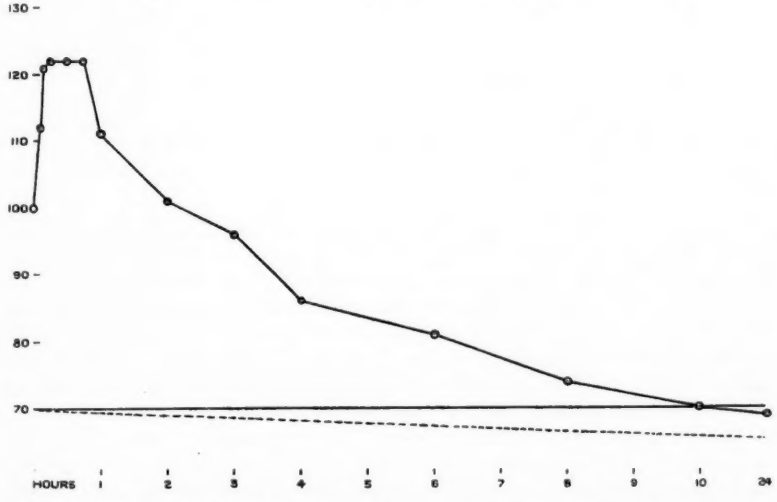

FIG. 5.—Injection of 12·8 c.c. per kg. of 15 p.c. gum in water observed for 24 hours.

osmometer through a cellophane membrane. In view of the property 15 p.c. gum acacia possesses in maintaining an increased blood volume observations were carried out on the duration of this raised blood volume, and from Fig. 5 it is noted that the blood gradually falls to normal 10 hours after the injection. That the kidneys play little part in this regulation in blood volume was proved by repeating this observation in an animal after bilateral nephrectomy, when a similar curve was obtained.

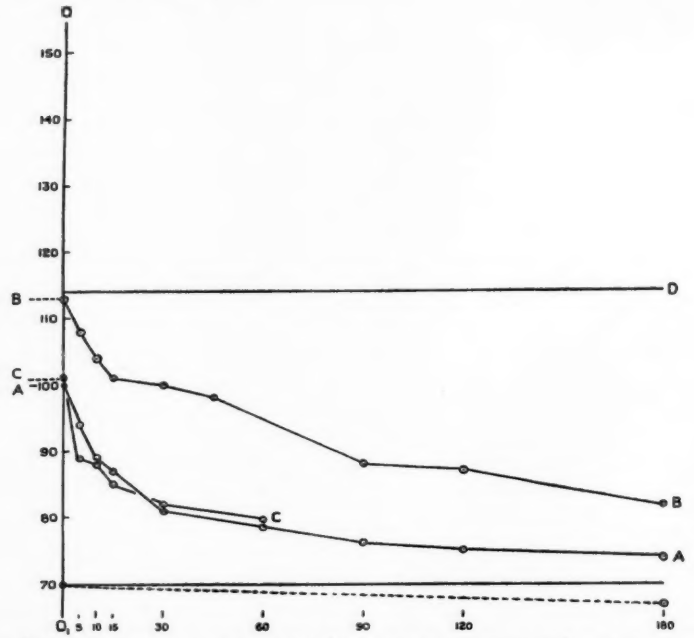

FIG. 6.—Injection of 44 c.c. per kg. of (A) blood serum ocmpared with B, 6 p.c. gum in 0·9 p.c. NaCl. C, Mean of readings for 2 p.c., 0·9 p.c., 0·6 p.c., 0·3 p.c. NaCl. D, Theoretical blood volume per kg. after injection of 44 c.c. per kg.

Experiment 5.—Fig. 6 shows the variations in the blood volume after the intravenous injection of blood serum. During the injection fluid leaves the vascular system for the tissues, and after the injection is completed the blood volume falls rapidly, although at the end of 3 hours it is still slightly above normal. For comparison there is reproduced the mean of the curves obtained with 2·0 p.c., 0·9 p.c., 0·6 p.c. and 0·3 p.c. NaCl (which were all found to be similar as demonstrated in Fig. 2), and it will be noticed how very closely these curves resemble one another. The curve of 6 p.c. gum acacia in 0·9 p.c. NaCl is also reproduced. Contrary to the views expressed by Drinker on the probable behaviour of gum acacia and serum when introduced into the mammalian circulation, it is noticed that the fall in blood volume is greater after the intravenous injection of serum than after the injection of gum, and

it seems probable that the blood proteins permeate the capillary membrane more easily than does gum acacia.

Experiment 6.—All the experiments described above on the behaviour of gum, blood-serum, and the various percentages of NaCl within the mammalian circulation have been carried out on the intact animal. But the reputed merit or demerit of these solutions lies in their ability to act as a substitute for blood after hæmorrhage and opinions still differ as to their relative values.

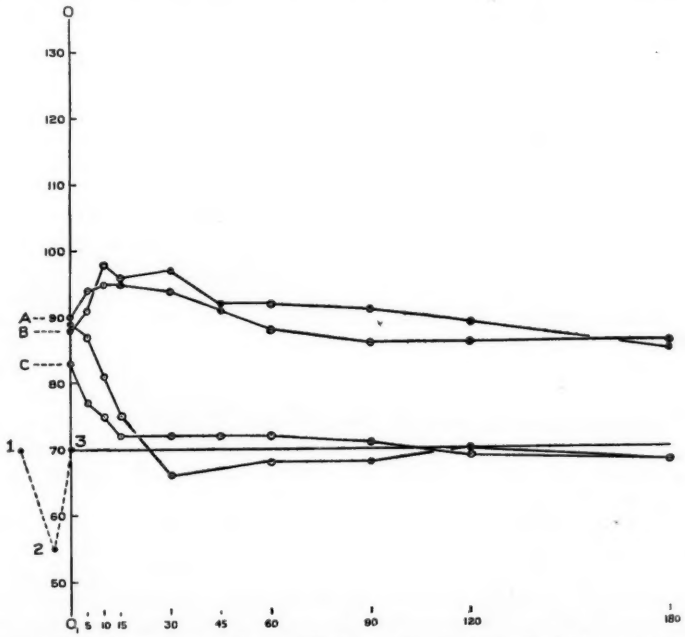

FIG. 7.—(1) Beginning of hæmorrhage. (2) End of hæmorrhage, beginning of injection. (3) End of injection. A, 6 p.c. gum, 0·9 p.c. NaCl. B, 15 p.c. gum aqueous. C, 0·9 p.c. NaCl. Blood serum (unlettered).

It was decided therefore to study the blood volume in animals when gum acacia, serum or NaCl was injected intravenously immediately after a controlled hæmorrhage equal to one-fifth of the total blood volume. These solutions were injected in amounts equal to the volume of blood removed by the hæmorrhage, so that the theoretical blood volume after blood-letting and infusion would equal the original blood volume. Fig. 7 shows the curves obtained. During hæmorrhage it has been shown (Robertson, 1935) there is a prompt and immediate transfer of fluid from the fluid stores throughout the body to the vascular system and to make good the deficit in blood volume. It is, however, apparent that during the injection of the infusion, fluid continues to be attracted from the tissues to the vascular system for the following

reasons: The post-hæmorrhage volume averaged 65 c.c. per kg. (and corrections were made to this figure where this was exceeded), indicating an entrance into the vascular system of 10 c.c. per kg. The infusion was equal to 15 c.c. per kg., and thus if no fluid entered or left the vascular system during the infusion the blood volume at its completion should be 65 c.c. + 15 c.c. = 80 c.c. per kg. In all cases this figure was exceeded thus :

Infusion.	Volume per kg.
0·9 p.c. NaCl	83 c.c.
Blood serum	89 ,,
15 p.c. gum in water	88 ,,
6 p.c. gum in 0·9 p.c. NaCl	90 ,,

After hæmorrhage equilibrium in the blood volume is practically established at the termination of the bleeding, so it would appear that all the infusions injected possessed the property of attracting tissue fluid and so behaving like hypertonic solutions. The progress of the blood volume after the infusion demonstrates that with 0·9 p.c. NaCl and blood-serum there is a steady loss of fluid, while with gum the fall in blood volume was inappreciable at the end of 3 hours. In the case of gum acacia the interchange of fluid differed very slightly whether the infusion was 15 p.c. gum in water or 6 p.c. gum in 0·9 p.c. NaCl.

DISCUSSION.

The fluid interchange has been studied after the intravenous injection of various concentrations of crystalloids, gum and blood-serum in the intact animal.

Crystalloid solutions.—Hypotonic, isotonic and 2 p.c. NaCl produced similar changes in the blood volume. It is surprising that 2 p.c. NaCl should fail to attract fluid from the tissues, and that the forces tending to expel fluid should exceed those bringing about an absorption. In the case of hypotonic solutions, one would have expected, by reason of the osmotic forces at play, that these solutions would have left the vascular system more rapidly than 0·9 p.c. NaCl, but such was not the case. Starling and Leathes have attempted to explain this paradox by suggesting that hypotonic solutions exert some influence on the vasomotor system, and that the effect of lowering the osmotic pressure of the blood may be to provide a greater accommodation for the fluid by increasing the capacity of the vessels. With very hypertonic solutions such as 33 p.c. NaCl or 50 p.c. glucose, and to a lesser extent 4 p.c. NaCl, the transudation of fluid entering and leaving the blood-stream was very rapid, and the osmotic attraction of these crystalloid solutions seemed solely to be governed by the actual time of the injection. As soon as the injection ceased so also did the power to attract fluid, and thereafter the excess of fluid was rapidly discharged back to the tissues again. The role the kidneys play in the immediate removal of fluid from the vascular system is negligible, for this interchange of fluid is unaffected by bilateral nephrectomy. The rates at which fluid enters and leaves the blood-stream are very rapid, and it

would seem that the filtering capacity of the capillary bed must be enormous to effect such transports of fluid.

Blood-serum.—It was found that blood-serum left the vascular system at a similar rate to that of isotonic NaCl. The injected serum was obtained from healthy cats and its average protein content was 6·2 gm. p.c. (with a colloid osmotic pressure of 30·9 cm. H_2O calculated from the albumen and globulin content as described by Govaerts (1925)), as compared with the recipient's serum of 6·35 gm. p.c. Similar changes in the blood volume to the above were obtained when human serum with a protein content of 7·4 gm. p.c., and a colloid osmotic pressure of 45 cm. H_2O was injected. It would appear therefore that the blood colloids, whether from the same animal or from another species, are not retained intravascularly when their injection causes an increase in the blood volume. The maintenance of a normal blood volume appears to be preserved by the loss of plasma from the blood-stream. This was confirmed in a subsequent experiment, when it was found that after the injection of 11 g. of protein (143 ml. of blood-serum) the total protein of the recipient's serum increased by only 2 g. Thus 9 g. of protein must have passed out into the tissue spaces along with the greater part of the fluid which was in excess of the normal blood volume. There was no increased urine output and there was no albuminuria.

Gum acacia.—It has been shown *in vitro* that the addition of NaCl to gum acacia depresses its osmotic pressure. Observations on the interchange of tissue fluid following the injection of 6 p.c. gum demonstrate that as increasing concentrations of NaCl up to 0·9 p.c. are added, so the greatest blood volume becomes less and less. In all experiments, however, 3 hours after the injection, the blood volume is still 20 p.c. above normal and greatest (30 p.c.) in the case of gum in water. Although NaCl depresses the osmotic pressure of gum, nevertheless gum given with NaCl does not leave the vascular system with the same ease as do the serum proteins. If hypertonic NaCl is added to gum, an attraction of fluid takes place during the injection greater than can be accounted for by either the gum or the NaCl alone, but after the completion of the injection fluid rapidly leaves the vascular system, and 3 hours afterwards the blood volume is less than in the case of gum and isotonic NaCl.

After a controlled hæmorrhage the behaviour of 0·9 p.c. NaCl, blood-serum and gum acacia was similar to that detailed in the intact animal, namely that blood-serum leaves the vascular system at the same rate as 0·9 p.c. NaCl, and that gum acacia is retained intravascularly to a greater extent than blood serum. These findings differ from those obtained in the frog by Drinker, who found that the capillaries of the frog were very permeable to gum in NaCl, but not to gum in NaCl plus horse-serum. It would appear that the capillaries of the frog and of the mammal differ, and Drinker's conclusions, " that gum as a useful injection material must depend more upon the company in which it finds itself when placed within the mammalian circulation than upon any significant suitability inherent in the gum ", is not supported.

When solutions of easily diffusible crystalloids are introduced into the mammalian circulation, it is well known that the osmotic pressure exerted by these injections is only temporary and lasts only as long as the injection. In the cases of colloids, however, these substances are not easily diffusible, and it

is believed that they can exert an osmotic pressure for some time after their introduction into the circulation. The response of the body, however, to these colloid injections differs a great deal from what one would expect as judged by their behaviour in an osmometer.

Volumetric changes in the mammalian circulation would appear to depend upon some other factor in addition to the role played by the osmotic pressure of blood, and the mechanism for the maintenance of fluid equilibrium cannot altogether be explained on purely physical grounds.

SUMMARY.

1. When hypertonic solutions such as 33 p.c. NaCl or 50 p.c. glucose are injected intravenously at the rate of 5 c.c. per kg. there is an enormous increase reaching often a maximum of double the original blood volume. The increase takes place very rapidly and only during the period of the injection. The moment the injection is stopped, the blood volume begins to fall rapidly and in about half-an-hour it is back to normal.

2. When 2 p.c. NaCl, 0·9 p.c. NaCl and 0·6 p.c. and 0·3 p.c. NaCl are injected intravenously, fluid leaves the vascular system during the injection, so that the blood volume immediately after the injection is completed is less than the theoretical level expected. After the injection the blood volume falls, but at the end of an hour it was still about 15 p.c. above normal.

3. The behaviour of an intravenous injection of blood serum is similar to that of either hypotonic, isotonic or 2 p.c. NaCl.

4. In the mammalian circulation the addition of NaCl to gum acacia depresses the power of the gum acacia to attract fluid. When the concentration of NaCl exceeds 1 p.c. then the attraction of fluid during the injection is greater than can be accounted for by either the gum or the NaCl alone.

5. In studying the blood volume changes of a cat after the intravenous injections of 6 p.c. gum acacia in various strengths of NaCl, it was found that 6 p.c. gum acacia in normal saline most nearly resembles blood serum.

6. After a hæmorrhage an injection of blood serum produces in the blood volume a similar response to that after an injection of 0·9 p.c. NaCl ; and in both the blood volume rapidly falls after the injection. Gum acacia on the other hand produces a very much slower fall in the blood volume.

7. Gum acacia is retained intravascularly to a greater extent than blood serum.

The author wishes to thank Prof. E. C. Dodds for his criticism and advice.

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CHEMOTHERAPEUTIC STUDIES IN THE ACRIDINE SERIES. THE RELATION BETWEEN CHEMICAL CONSTITUTION AND BIOLOGICAL ACTION IN SIMPLE AMINOACRIDINES.

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THE familiar acridine antiseptics are, according to Browning, characterized by three properties peculiar to them : they act adversely in extreme dilution towards the majority of pathogenic bacteria, their action is selective in that mammalian tissues are relatively unharmed in these dilutions, and lastly their antiseptic action is undiminished, in some cases even increased, in the presence of serum.

The first study of the relation between antiseptic action and chemical constitution among acridine derivatives was made by Browning *et al.* (1922). They demonstrated that the antiseptic action of these substances depended upon an intact acridine nucleus, and that the presence of an amino substituent was required for the development of the maximum effect. The majority of the experimental work since undertaken has dealt with derivatives of more complicated structure than Browning's simple aminoacridines and, of the many substances since elaborated, only rivanol (2 : 5-diamino-7-ethoxyacridine) has enjoyed a measure of success. Meanwhile the simpler acridine derivatives which would lend themselves far better to the study of the relationship between structure and action received no further attention. Accordingly the work here described was undertaken to make some contribution to the fundamental chemotherapy of the acridine antiseptics, partly through the bacteriological comparison of substances already known, but principally through the synthesis and testing of new compounds. It is unsafe to assume that the 2-position has a special significance, since the more difficultly accessible isomerides have scarcely received attention. Of the 20 possible diaminoacridines, only proflavine (Fig. 1*b*) has been thoroughly investigated, although Bogert *et al.* (1930)

made some preliminary tests with 3 : 7-diaminoacridine and Morgenroth *et al.* (1921) carried out tests, restricted to streptococci, with the 2 : 5-isomeride. Proflavine, the simplest known entity possessing the full characteristic activity, forms the natural starting-point for the present investigations, which consequently fall into two divisions :

(i) Modification of the molecule of proflavine (Fig. 1*b*), with the object of discovering whether, provided a single 2-amino group be retained, the activity is susceptible to considerable change. In this connection the preparation of

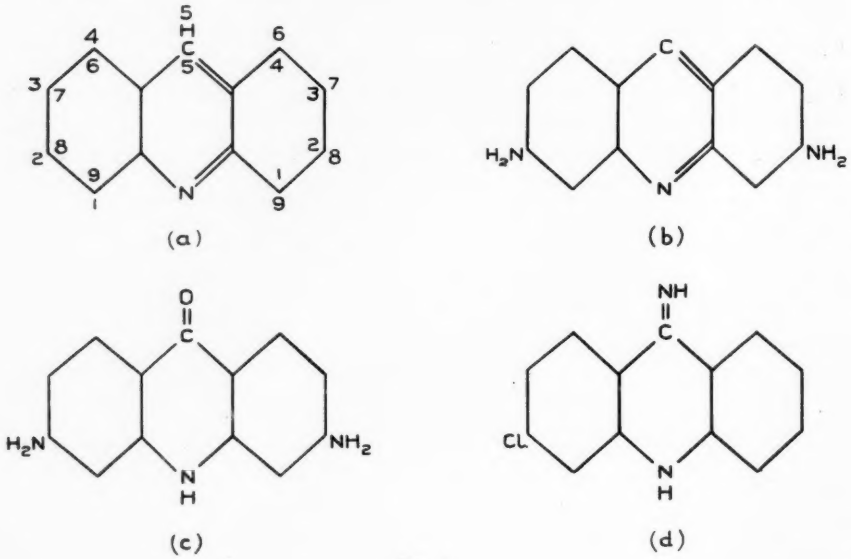

FIG. 1.

2 : 8-diaminoacridone (Fig. 1*c*), 2-amino-8-acetamidoacridine and 2-amino-acridine have been undertaken.

(ii) Preparation of the isomerides of proflavine to obtain information concerning the optimum positions of the amino group(s). In this connection it should be realized that the acridine molecule has virtual symmetry, and as there are only five different positions for substitution (*cf.* Fig. 1*a*), each of these may be explored by the preparation of a limited number of compounds. The same phenomenon gives rise to dual numbering.

The substances required were prepared (Albert and Linnell, 1936), and tested, those marked with an asterisk in Table I being new to chemical literature. The general method of preparation was to take a chloro-benzoic acid and an aminobenzene, both appropriately substituted, and to condense these by the Jourdan-Ullmann reaction to a nitro-diphenylamine-2-carboxylic acid. Ring closure with phosphorus oxychloride led to the corresponding nitro*meso*-chloroacridines often as a mixture of similar substances requiring scrupulous

TABLE I.

Substance.	Fluorescence in alcohol.	Fluorescence of hydrochloride in dilute aqueous solution.	Melting-point (Cent.).	Colour.	Colour of salts.	Solubility of base in boiling alcohol.
Acridine	Blue	Faint blue	111°	White	Yellow	Very soluble.
1-aminoacridine	None	None	105°	Yellow	Brown-orange	,, ,,
2- ,,	Yellow-green	Yellow-green	219°	,,	,,	,, ,,
*4- ,,	None	None	181°	Brown-orange	Purple	,, ,,
5- ,,	Blue-green, turned to yellow-green by trace of alkali	Bright violet	234°	Yellow	Cream	,, ,,
*1 : 3-diaminoacridine	None	None	225°	Orange	Purple	,, ,,
*1 : 7- ,,	,,	,,	126°	Orange-yellow	,,	,, ,,
*2 : 5- ,,	Yellow-green	Yellow-green	141°	Yellow	Cream	,, ,,
*2 : 6- ,,	None	None	213–6°	Brown-orange	Red	,, ,,
*2 : 7- ,,	Yellow-green	,,	355°	Orange-yellow	Ruby to purple	Slightly soluble.
2 : 8- ,,	,,	Yellow-green	281°	Yellow	Scarlet	Very soluble.
*2 : 9- ,,	None	None	249°	,,	Red	,, ,,
*2-amino-8-acetamidoacridine	Yellow-green	Yellow-green	286°	Orange-yellow	Scarlet	,, ,,
*5-amino-2-chloroacridine	As 5-aminoacridine	Bright violet	272·5°	Pale yellow	Cream	,, ,,
*3 : 7-diaminoacridine	Yellow-green	None	315–8°	Orange-yellow	Purple	Slightly soluble.

Dilute solutions of the hydrochloride have been specified as these salts do not fluoresce in concentrated solutions.

fractional separation. Hydrolysis produced the appropriate nitroacridones which were reduced in stages, first to the aminoacridones and then to the aminoacridines. In the case of compounds containing *meso*amino groups, these were obtained by the action of ammonia under pressure at the *meso*-chloroacridine stage. 1 : 3-diaminoacridine could not be obtained by the general method, but was formed from the reduction of the corresponding dinitroacridine obtained from anthranilic aldehyde. 2-amino-8-acetamido-acridine was made by the regulated acetylation of proflavine.

The chemical and physical properties of these substances are of considerable interest in that they prove to be closely related to the molecular structure. The properties are set out in Table I, in which the remaining monoamino-acridines have been included for the sake of completeness.

It has been believed up to the present that all acridine compounds fluoresced in solution, but, as will be seen from Table I, all acridines containing a 1- or 4-amino substituent show no fluorescence in solution even in the presence of a substituent usually accompanied by strong fluorescence such as the 2-amino group. On the other hand the 2-amino substituted acridines alone have hydrochlorides fluorescing green. The fluorescences of the 5-amino group are quite individual, and suggest that in the case of " 5-aminoacridine " and " 5-amino-2-chloroacridine " no primary amino group is actually present and that, in the form of their salts at least, they assume the tautomeric form illustrated by Fig. 1*d*, where 5-amino-2-chloroacridine is shown as the isomeric 2-chloroacridonimine. In support of this contention may be mentioned the fact that these two compounds do not diazotise. 2 : 5-diaminoacridine, possessing a genuine primary amino group in the 2-position, gives the normal fluorescences of the 2-amino compounds, but it shares two properties in common with the other 5-amino compounds, namely, its salts are paler in colour than the base—quite an exceptional property in an amino dyestuff series—and they are precipitated by very small amounts of sodium chloride. It is therefore reasonable to assume that this compound also possesses the acridonimine and not the acridine structure.

METHODS OF BIOLOGICAL TEST.

The majority of these compounds have been submitted to tests of toxicity for mammalian tissues, and of capacity to destroy or restrain the growth of bacteria. Most of these observations are here reported for the first time, but tests of toxicity as indicated by the lethal dose for mice given by subcutaneous injection are reported in full elsewhere by Albert, Dyer and Linnell (1938). Their results for the more promising of these compounds are quoted in Table VI. Toxicity has also been studied by observing action on leucocytes by two methods, and antiseptic capacity has been estimated by tests of bacteriostatic and bactericidal action.

The standard substance by which the results should be judged is proflavine, since this is a single substance obtainable in a state of purity. Acriflavine is included in some experiments, and appears in one series to exceed all other compounds tested in antiseptic activity. For this sample of acriflavine this

may be true, but it has been shown by Gailliot (1934), Marshall (1934), Reimers (1935), and Hall and Powell (1934, 1936), that acriflavine from different sources may vary, as it is a mixture of two compounds (2 : 8-diaminoacridine hydrochloride and its methochloride) in variable proportions, with other substances which tend to increase their solubility. It is therefore an ill-defined and variable product, and whatever its merits, cannot serve as a standard by which to judge other compounds.

Action on Bacteria.

The following tests of bacteriostatic and bactericidal action were designed to afford general indications of the susceptibility of bacteria to these compounds, and pretend neither to extreme accuracy nor to finality.

Tests of bacteriostatic action.—Sets of tubes of broth containing varying concentrations of the antiseptic were inoculated with 0·02 c.c. of a 24-hour broth culture of each of five bacteria, and incubated at 37° C. for 48 hours, after which the presence or absence of visible growth was noted. A second set of tubes contained in addition 10 p.c. of sterile sheep's serum. The dilutions of the antiseptic in the medium were as follows; their numbers are those by which they are denoted in Table II:

Dilution 1 :	1 in 5,000	Dilution 6 :	1 in 160,000
„ 2 :	1 in 10,000	„ 7 :	1 in 320,000
„ 3 :	1 in 20,000	„ 8 :	1 in 640,000
„ 4 :	1 in 40,000	„ 9 :	1 in 1,280,000
„ 5 :	1 in 80,000		

The five bacteria used were *Streptococcus pyogenes* (2 strains), *Staphylococcus aureus*, *Bact. coli* and *Ps. pyocyanea*. In Table II, which gives the results of these tests, those obtained with *Ps. pyocyanea* are omitted, since this organism grew freely in every dilution of each antiseptic.

In such simple tests, using a widely-spaced series of dilutions, too much reliance is not to be placed on the significance of any single result, but by averaging a large number, a useful comparison can be made. By totalling the results for each compound as given in Table II, a figure is obtained which serves as an index of general bacteriostatic capacity. These figures are given in the last column of Table II only in the case of nine compounds which were fully investigated. Of the remainder, three have clearly little or no antiseptic value, and one, 5-amino-2-chloroacridine monohydrochloride, had in the meanwhile been found in other experiments to be considerably more toxic than the remainder, and its action on bacteria was therefore not studied further.

On examining these results in more detail, it is clear that *Streptococcus pyogenes* is the most susceptible of the bacteria used, and Strain 2, which was recently isolated, is more susceptible than Strain 1, which had been kept in the laboratory for several years. It is perhaps idle to study the bearing of these results on the contention of Browning *et al.* (1917, 1922) that serum may increase the antiseptic activity of acridine compounds, since the technique employed is widely different. In these experiments the minimum bacteriostatic concentration is often the same whether the broth contained serum or not; in 6 cases only is bacteriostatic activity greater in serum broth, two being

experiments with *S. aureus* and four with *Bact. coli* (the bacteria used by Browning), while this result is never observed in an experiment with *Streptococcus pyogenes*. On the other hand, in 15 instances, 10 being experiments with *Streptococcus pyogenes*, the addition of serum appears to have reduced bacteriostatic activity.

TABLE II.—*Minimum Concentrations Completely Preventing Visible Growth in 48 Hours at 37° C. (for key to figures see text).*

Compound.	Broth.				Broth + 10% serum.				
	Strep. pyogenes 1.	*Strep. pyogenes* 2.	*Staph. aureus.*	*Bact. coli.*	*Strep. pyogenes* 1.	*Strep. pyogenes* 2.	*Staph. aureus.*	*Bact. coli.*	Totals.
1 : 3-diaminoacridine monohydrochloride	—	0	0	0	—	0	0	—	—
1 : 7- „ „	—	0	0	0	—	0	0	—	—
2-aminoacridine monohydrochloride	5	6	4	5	4	5	3	4	36
2 : 5-diaminoacridine monohydrochloride monohydrate	4	7	3	3	2	7	2	3	31
2 : 6 „ hemisulphate	4	5	4	5	4	5	4	5	36
2 : 7- „ „	4	5	3	4	4	5	4	5	34
2 : 7- „ monohydrochloride	—	5	4	4	—	5	4	—	22
2 : 8- „ hemisulphate (proflavine)	6	7	4	4	4	6	4	5	40
2 : 9- „ „	0	4	0	0	0	0	0	0	4
2-amino-8-acetamidoacridine monohydrochloride	2	5	1	2	1	5	2	4	22
3 : 7-diaminoacridine hemisulphate	3	3	4	2	2	2	3	2	21
5-amino-2-chloroacridine monoacetate	—	7	6	6	—	6	5	—	—
2 : 8-diaminoacridone monohydrochloride	—	0	0	0	—	0	0	—	—
Acriflavine	5	9	5	5	5	8	5	6	48

Tests of bactericidal action.—The four most promising of the new compounds were submitted to a test of bactericidal action, in which proflavine was used as the control substance. In these experiments the rate of destruction of *Streptococcus pyogenes* in a medium containing 50 p.c. of blood was determined as follows :

A mixture was made of 2·5 c.c. sterile defibrinated human blood, 1·5 c.c. of a 1 in 20 dilution in Ringer's solution of a 24-hour broth culture of *Streptococcus pyogenes*, and 1 c.c. of a 1 in 1000 solution of the compound to be tested ; the concentration of the latter in the mixture was thus 1 in 5000 (0·02 p.c.). This mixture was placed in a water-bath at 37° C., and at 30-minute intervals for 3 hours 0·5 c.c. was removed and added to 49·5 c.c. sterile Ringer's solution in a flask ; from this 1 in 100 dilution a second 1 in 100 dilution was similarly prepared, and from each of these 1 c.c. was shown as a shake culture in a blood-agar plate. In one or other of these plates the number of resulting colonies was always such as could be counted. The control tube contained the same ingredients with the exception that 1 c.c. of Ringer's solution took the place of the antiseptic ; an immediate culture from 1 c.c. of a 1 in 10,000 dilution from this tube always gave a count of about 150 colonies, while at the end of the 3 hours the streptococci in this tube had always multiplied considerably.

In Table III are given the results of two such experiments.

TABLE III.—*Numbers of Surviving Bacteria per c.c. in Mixtures at 37° C. containing 50 p.c. of blood, 0·02 p.c. of the Antiseptic, and an Inoculum of* Streptococcus pyogenes.

	Time						
	Zero (control).	30 minutes.	60 minutes.	90 minutes.	120 minutes.	150 minutes.	180 minutes.
Experiment 1:							
Proflavine	1,480,000	1,250,000	690,000	240,000	40,000	20,000	6,700
2-aminoacridine monohydrochloride	1,480,000	1,850,000	1,290,000	1,040,000	960,000	440,000	460,000
2 : 5-diaminoacridine monohydrochloride	1,480,000	130,000	10,000	5,400	1,600	300	0
Experiment 2:							
Proflavine	1,630,000	1,850,000	1,950,000	640,000	100,000	58,700	4,500
2 : 5-diaminoacridine monohydrochloride	1,630,000	110,000	50,000	11,800	8,200	3,800	1,900
2 : 6- „ hemisulphate	1,630,000	1,760,000	1,840,000	1,430,000	980,000	610,000	280,000
2 : 7- „ monohydrochloride	1,630,000	1,580,000	1,880,000	430,000	130,000	29,300	6,100

Minor irregularities in the counts obtained are to be expected from the nature of the method, although the apparent increase in the count during the first hour, of which there are three examples, is probably genuine, and represents bacterial multiplication before antiseptic action has taken effect. The order of activity is not the same as in tests of bacteriostasis ; here the 2- and 2 : 6 compounds are the least active, and the 2 : 5 emphatically the most active in both experiments, this being the only one to achieve a substantial reduction in bacterial content within the first half-hour. Proflavine and the 2 : 7-compound almost achieve complete sterilization within 3 hours, but their action is slower, only taking marked effect after 60 or 90 minutes. Rapidity of action is therefore another property which varies among these compounds with chemical structure, and it is this factor which explains differences in the order of activity as defined by the two types of test. It is clearly necessary that in any further study of these compounds their reaction velocities should be studied by a more accurate method ; this would involve, besides other differences in technique, the employment of some other micro-organism than *S. pyogenes*. All such experiments with streptococci are probably liable to error owing to the uncontrollable disruption of chains of cocci.

Toxicity to Human Leucocytes.

Preliminary tests with phagocytosis experiments using Wright's opsonic index technique (Wright and Colebrook, 1921) showed that if phagocytic mixtures were made with equal parts of washed blood-cells, fresh serum, suspension of staphylococcci, and of a 1 in 1050 solution of the compound in physiological saline (giving a final concentration of 1 in 4200), no significant inhibitory effect could be observed. Except for 2 : 5 and 2 : 7 diaminoacridine hydrochlorides and 2 : 8 diaminoacridine sulphate, stronger solutions than 1 in 1000 in physiological saline could not be used, as the antiseptics were precipitated.

Phagocytosis experiments were therefore carried out with these substances only (Table V). As compared with 2 : 8 diaminoacridine hemisulphate (proflavine), 2 : 5 diaminoacridine hydrochloride appeared to be less inhibitory, and 2 : 7 diaminoacridine hydrochloride more so.

Experiments were carried out to test the continued motility of polymorphonuclear leucocytes in various concentrations of the compounds. Specially prepared grease-free slides and coverslips were used, as for supra-vital staining of leucocytes. One 3 mm. loopful of blood (A. E. F—'s) and one loopful of the antiseptic solution in saline were mixed on a $\frac{7}{8}$-in. square coverslip and a slide gently lowered over the mixture. This immediately spread to form a uniform film. The side was turned face upwards and the edges of the coverslip waxed round with low melting-point wax (45° C.). A saline control was put up with each batch, and the procedure was rapid to prevent evaporation. Each preparation was examined in a thermostatic microscope hot box at 38° C. In each case the control preparation showed active motility at the end of the experiment. Observations were made at 30, 60 and 90 minutes.

The results are given in Table IV, the 60-minute readings only being

recorded, since these are representative. It was interesting to observe that with acriflavine and proflavine, the polymorphonuclears showed bright yellow diffuse staining, and despite this they retained active motility. In fact these were the only two compounds tested with which a moderate degree of motility was still maintained after 90 minutes in a final concentration of 1 in 2000. With the other compounds slight staining only, or none at all, was seen.

2 : 5 diaminoacridine hydrochloride had a marked effect on motility, and showed a tendency to crenate and agglutinate red blood-cells in the motility tests, and to agglutinate staphylococci in the phagocytosis experiments. This fact and the shorter time and higher concentrations of the compounds used in the phagocytosis experiments probably explain the different results shown by the two methods.

TABLE IV.—*Motility of Polymorphonuclear Leucocytes in Solutions of Acridine Compounds.* (*At 60 minutes.*)

Final concentration of compound.	1 in 2100.	1 in 4200.	1 in 8400.	1 in 16,800.
2-aminoacridine HCl	0	0	±	/
2 : 5-diaminoacridine HCl	0 (A)	0 (B)	±	+
2 : 6- ,, sulph.	0	±	+	/
2 : 7- ,, ,,	± (P)	+	++	/
2 : 7- ,, HCl	±	+	++	/
2 : 8- ,, sulph. (proflavine)	+	+	++	/
2 : 9- ,, sulph.	+	++	++	/
2-amino-8-acetamido-acridine monohydrochloride	0	±	+	/
5-amino-2 chloroacridine acetate	0	0	0	/
Acriflavine	±	±	++	/
	1 in 2000	1 in 4000	1 in 8000	
Phenol	0	±	+	/

0 = Non-motile. ± = Sluggish. + = Moderate activity. ++ = As control. (A) R.B.C. crenated and agglutinated. (B) = R.B.C. crenated. (P) = Partial precipitation of dye. / = Observation not made.

TABLE V.—*Phagocytosis of Staphylococci by Polymorphonuclear Leucocytes in the Presence of Acridine Compounds.*

Final concentration of compound.	Percentage of control.		
	1 in 1000.	1 in 2000.	1 in 4000.
2 : 5-diaminoacridine HCl	34	72	97
2 : 7- ,, ,,	14	31	62
	1 in 1200.		
2 : 8- ,, sulph. (proflavine)	36	41	64

DISCUSSION.

In interpreting the results of these experiments two matters are of interest: the relation between chemical structure and biological action, and the assessment of the relative merits of these compounds as antiseptics for clinical use.

In connection with the former, the following conclusions may be drawn:

1-aminoacridines: These are all devoid of antiseptic activity, even though a strongly acting antiseptic substituent, such as the 2-amino group, be present also. They show a comparatively low mammalian toxicity provided two amino groups are not in the same ring.

2-aminoacridines: The 2-amino substituent introduces marked antiseptic properties (see Browning, 1922, for comparative weak antiseptic action of unsubstituted acridine), which are further developed by the addition (in another ring) of a 2-, 3-, 4-, or 5-amino group. This leads to increased toxicity only when the added substituent is a 2-amino group (giving proflavine: 2 : 8-diaminoacridine). Acetylation of one amino group in the latter compound leads again to loss of activity, but the effect produced is qualitatively distinct from that of the monoamino compound, in that the latter is depressed by serum, whereas the former is increased thereby.

3-aminoacridines: 3 : 8- (*i. e.* 2 : 7-) diaminoacridine is approximately equal to proflavine in bactericidal activity whilst possessing a much lower mammalian toxicity. However, when more than one 3-amino group is present (as in 3 : 7-diaminoacridine) the activity decreases.

4-aminoacridines: Three such compounds have been prepared, viz. 4-amino-, 4 : 7-, and 4 : 8 (*i. e.* 2 : 6)-diaminoacridines have been prepared, but only the last-named has yet been submitted to bacteriological tests. It showed considerable activity.

5-aminoacridines: These display high antiseptic activity, particularly against streptococci. 5-amino-2-chloroacridine is the most active of all the new compounds so far investigated, but its mammalian toxicity is unfortunately increased in like proportion.

Acridones: The acridone nucleus does not share the antiseptic properties of the acridine nucleus.

To assess the relative merits of these compounds as practical antiseptics it is necessary to take a number of factors into account, and the whole of these have been assembled in Table VI, including data on solubility which have not previously been given, and on toxicity as indicated by the lethal dose given subcutaneously to mice (Albert, Dyer and Linnell, 1938); the remaining data are as previously stated, except that bactericidal power is expressed as the percentage of the total inoculum of *S. pyogenes* killed in 2 *hours* under the conditions of the experiments detailed in Table IV.

Lack of solubility in the presence of sodium chloride is a grave disadvantage to use *in vivo*, and four of the compounds named in Table VI fail for this reason. Among the properties of the remainder it will be seen that the degrees of toxicity indicated by the two methods used do not correspond. Whereas acriflavine and proflavine are the least toxic to leucocytes, they are the most toxic as judged by the lethal dose for mice. Since, despite this lethal action, acriflavine is not known to cause toxic effects when locally

TABLE VI.—*Physical and Biological Properties of Nine Acridine Compounds.*

	Solubility in water at 15° C.	Solubility in physiological saline.	*Lethal dose for mice in gm. per kgm. of body-weight.	Maximum concentration permitting motility of leucocytes (see Table IV).	Index of bacteriostatic capacity (see Table II).	Percentage of *S. pyogenes* killed by 1 in 5000 solution 2 hrs. (see Table III).
2-aminoacridine monohydrochloride	0·5%	Rather less	0·35	1 in 8400	36	30
2 : 5-diaminoacridine monohydrochloride	1%	0·4%	0·3	1 in 8400	31	99·5–99·9
2 : 6- „ hemisulphate	0·4%	Only slightly soluble	0·35	1 in 4200	36	40
2 : 7- „ monohydrochloride	1%	0·4%	0·5	1 in 2100	34	92
2 : 8- „ hemisulphate (proflavine)	0·5%	0·3%	0·2	1 in 2100	40	93·7–97·3
2 : 9- „ „	1%	Only slightly soluble	0·75	1 in 2100	4	Not tested.
2-amino-8-acetamidoacridine monohydrochloride	1%	Ditto	0·38	1 in 4200	22	„ „
5-amino-2-chloroacridine monohydrochloride	Over 2 %	Almost insoluble	0·13	1 in 8400	High (experiments incomplete)	„ „
Acriflavine	Very variable	0·2%	0·05	1 in 2100	48	„ „

* Albert, Dyer and Linnell, Quart. J. Pharm., 1938, 649.

applied, it is unlikely that the use of any of these substances as local antiseptics would be followed by undesirable general effects. On the other hand, if the capacity of acridine compounds to prevent wound infection depends in part on the fact that leucocytic activity continues in their presence, then the degree of their interference with such activity must affect their usefulness. 2 : 7-diaminoacridine hydrochloride is the only one of the new compounds in Table VI which is as innocuous to leucocytes as acriflavine and proflavine. Since this compound also possesses a bactericidal capacity approximately equal, and a bacteriostatic capacity scarcely inferior, to that of proflavine, it emerges from these tests as the most likely of the newly synthesized compounds to prove useful therapeutically.

SUMMARY.

1. Ten new acridine derivatives have been synthesized, the properties of which are described.

2. These have been submitted to tests of bacteriostatic action on *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bact. coli* and *Ps. pyocyanea*, and certain of them to tests of bactericidal action on *Streptococcus pyogenes*, proflavine being used as the control substance.

3. *Streptococcus pyogenes* is the most susceptible of these four bacteria to the action of acridine compounds, and *Ps. pyocyanea* is entirely insusceptible to all of them.

4. The belief that serum enhances the action of acridine compounds on bacteria receives little support from these observations.

5. Toxicity was tested by determining the lethal dose for mice and the maximum concentration tolerated by human leucocytes ; these methods give divergent results.

6. Several well-marked relationships between chemical structure and biological action have been established.

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THE CROSS REACTIONS OF VEGETABLE GUMS WITH TYPE II ANTIPNEUMOCOCCAL SERUM.

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THE reaction of horse antisera to pneumococci, Types II and III, with gum acacia, was described by Heidelberger, Avery and Goebel (1929). Horse antisera to Type II pneumococci also form precipitates with various other vegetable gums. These reactions have been investigated for the information that may be obtained about cross reactions. A short account has appeared in the *Journal of the Society of Chemistry and Industry* (Carpenter and Marrack, 1934).

METHODS.

When gum acacia is weakly hydrolyzed by hydrochloric acid at room temperature pentose is split off. Heidelberger, Avery and Goebel (1929) found that the gum, treated in this way, would form a precipitate in higher dilutions than would the natural gum, when added to antipneumococcal sear Types II and III.

The gums that we have investigated have been treated in this way; the products are called "weakly hydrolyzed gums"; but the name of the gum will be used in this paper to mean these weakly hydrolyzed products, unless a special note is made to the contrary.

The weakly hydrolyzed products were further hydrolyzed by boiling water with N. H_2SO_4 for 2 to 3 hours. The H_2SO_4 was precipitated with $Ba(OH)_2$ and the fluid neutralized by boiling with excess of $BaCO_3$ or $CaCO_3$. The excess of carbonate was removed and the Ba or Ca salt of the acid fraction precipitated with alcohol. The neutral fraction was obtained from the supernatant fluid by evaporation under reduced pressure. The Ba and Ca salts were freed from neutral sugars by repeated solution in water and precipitation with alcohol.

Uronic acid was estimated by a method resembling that of Burkhart, Bauer and Link (1934) but quantities of about 0·4 gm. were used.

Aldehyde was estimated by the method of Goebel (1927).

In measurements of antibody precipitation, the polysaccharide was added to the antiserum at 0° to 5° C. and well mixed; the mixture was kept at 0° C. overnight, centrifuged, and the precipitate washed twice with 0·9 p.c. NaCl solution. The nitrogen in the precipitate was estimated by incineration with H_2SO_4 and perhydrol and Nesslerization.

Chemical Composition.

The composition of 4 of the gums under consideration has been investigated thoroughly. The supposed structures are shown in Table I. These gums consist of an acidic nucleus to which are attached various sugar units ; in 3 of the 4 gums the nucleus contains glucuronic acid or methyl glucuronic acid.

TABLE I.—*The Structure of Four Polysaccharides.*

Cherry gum . . . Butler and Cretcher (1931)	Glucuronic acid | Glucuronic acid | Mannose | Mannose | Mannose | 6 (Galactose) | 6 (Xylose) 8 (Arabinose)	Acid nucleus. Pentose split off by HCl in the cold.
Gum acacia . . . Butler and Cretcher (1929) Heidelberger (1929) Challinor, Haworth and Hirst (1931)	Glucuronic acid | Galactose | 2 (Galactose) | 3 (Arabinose) 1 (Methyl pentose)	Acid nucleus. β-glucurono–6-galactose. About half of pentose split off by HCl in the cold.
Mesquite gum . . . Anderson and Otis (1930)	Methoxy-glucuronic acid | Galactose | 2 (Galactose) | 4 (Arabinose)	Acid nucleus. Pentose split off by HCl in the cold.
Flax-seed mucilage . . Hilger (1903) Anderson and Crowden (1930)	Galacturonic acid | l-Rhamnose | Glucose Galactose Xylose Arabinose	Acid nucleus.

Apricot gum, studied by Van der Haar (1920), contains 10·5 p.c. of uronic acid, 33·5 p.c. of galactose and 57·5 p.c. of pentose and methyl pentose in the water-free sample. The uronic acid was not identified ; it was not galacturonic acid and probably not glucuronic acid. Gum tragacanth was shown by Van der Haar (1920) to contain 58 p.c. of pentose and methyl pentose, 32·8 p.c. of galacturonic acid, 3·8 p.c. of galactose and 2·2 p.c. of glucose ; glucuronic acid was not present. Norman (1931) has found that the more soluble part of this gum contains 3 molecules of galacturonic acid to 3 molecules of arabinose ; the pentose molecules are split off progressively with boiling with sulphuric acid.

The equivalent weight of the product obtained by us after the hydrolysis of plum gum with hydrochloric acid in the cold, was 710, with a uronic acid content corresponding to an equivalent weight of 660. As the aldehyde content was less than 1 per 2000, the molecular weight must have been higher than this. After hydrolysis with boiling N. sulphuric acid for 3 hours, galactose and the Ba salt of an acid were obtained. The equivalent weights of this acid, calculated from the Ba and uronic contents of the salt were 346 and 339 and the molecular weight from the aldehyde estimation was 440. Attempts to prepare this acid in a crystalline form either free or as the salt of an organic base failed. Neither mucic nor saccharic acids were found after oxidation with nitric acid, so the uronic acid does not appear to have been galacturonic or glucuronic acid.

Our samples of gum ghatti were assumed to be the gum of the tree Anogeissus Latifolia. However, we have no justification for assuming that the gum was pure. The equivalent weight of the product of hydrolysis by HCl in the cold was 730, with a uronic acid content corresponding to an equivalent weight of 630. One aldehyde group was contained in a weight of approximately 1700 so that the molecule contained about 3 equivalent weights. On further hydrolysis with boiling H_2SO_4 galactose was obtained and the Ba and Ca salts of an acid. Several specimens of these salts were prepared; and the Ba or Ca and the uronic acid contents of the majority of these salts corresponded with those of acids with equivalent weights between 365 and 450. In some specimens corresponding figures for the aldehyde were found ; but in others the aldehyde was too low, suggesting that compounds of higher molecular weight had not been removed completely. Attempts to obtain an acid of lower molecular weight by further hydrolysis with H_2SO_4 and with alcoholic HCl were not successful. Crystalline preparations were not obtained, nor were products, whose uronic acids and aldehyde contents were in better agreement, obtained by fractional precipitation with alcohol. Neither saccharic nor mucic acid were identified after oxidation with HNO_3 or with HBr and Br ; our supposition (Carpenter and Marrack, 1934) that we had obtained saccharic acid proved to be incorrect. On one occasion only a product with the melting-point and nitrogen content of mannosaccharic acid diamide was obtained ; and this was not obtained in other attempts, it may have been produced from an impurity of this sample of gum. The uronic acid therefore is neither glucuronic, galacturonic nor mannuronic acid.

An acidic nucleus, to which various sugar units are attached, is a common feature of these gums. This nucleus which is relatively resistant to hydrolysis

appears to contain a uronic acid and a sugar unit (equivalent weight 326 if the sugar is a pentose, 356 if the sugar is a hexose), except in the case of cherry gum in which the nucleus contains the glucuronic acid units and a hexose.

Hydrolysis by hydrochloric acid in the cold splits off pentose from acacia, cherry, plum, apricot, mesquite and ghatti gums. About half the pentose is split off from acaica gum (Heidelberger, Avery and Goebel, 1929) but all from cherry gum (Butler and Cretcher, 1931).

Quantitative Aspects.

Limits of precipitation.—With a strong antiserum 8211, used undiluted, the various gums formed a precipitate up to the limiting dilutions shown in Tabe II. We did not find precipitate formation with such extreme dilutions of gum acacia as did Heidelberger, Avery and Goebel (1929). As we did not use ring tests our tests were not so delicate. Butler and Cretcher (1931) report that Dr. Koch found that neither natural nor weakly hydrolyzed cherry gum formed precipitates with antisera to Type I, II and II pneumococci. There is, however, no doubt, that our samples of wild cherry gum were genuine, as they were obtained by one of us from the actual trees. In all cases hydrolysis with acid in the cold extended the range of precipitation.

TABLE II.—*Limiting Concentrations, Optimum Proportions and Maximum Nitrogen Precipitated from Sera.*

Polysaccharide.	Limiting concentration of polysaccharide forming a precipitate with serum 8211.	Optimum proportion in mg. per 1 c.c. of serum.			Maximum nitrogen in mg., precipitated by polysaccharide from 1 c.c. of serum.		
		Serum 8211.	Serum 16586.	Serum 16652.	Serum 8211.	Serum 16586.	Serum 16652.
S.S.S. II	..	0·30	0·44	0·175	1·29	1·55	0·70
Cherry gum; natural	No precipitate	..	..	..	..	..	..
„ „ weakly hydrolyzed	$1/10^6$	0·26	0·26	..	0·54	0·69	..
Acacia gum; weakly hydrolyzed	$1/2 \times 10^5$	0·8	0·8	0·26	0·285	0·34	0·19
Plum gum; natural	$1/2 \times 10^4$	..	..	..	..	..	..
„ „ weakly hydrolyzed	$1/2 \times 10^5$	ca 0·1	0·2	..	0·425	0·48	..
Apricot gum; weakly hydrolyzed	$1/10^5$	..	0·2	..	..	0·40	..
Ghatti gum; natural	$1/2 \times 10^4$	..	..	..	..	..	..
„ „ weakly hydrolyzed	$1/10^5$	0·5	0·4	..	0·37	0·33+	..
Mesquite gum; weakly hydrolyzed	$1/10^5$	2·5 to 6	6·5	..	0·285	0·33	..
Flax mucilage; weakly hydrolyzed	$1/2 \times 10^4$	..	6·6	..	..	0·37	..
Tragacanth gum; weakly hydrolyzed	$1/2 \times 10^4$	..	..	..	..	..	..

Zone phenomenon.—The amount of precipitate formed by adding gum to a constant amount of antiserum rises to a maximum with increasing amounts of gum added and then falls off. This was found with acacia, cherry and mesquite gum; it is illustrated in Table III.

TABLE III.—*Precipitation of Nitrogen from Sera by Increasing Amounts of Gums.*

	Amount added; mg. per 1 c.c.	Mg. of nitrogen precipitated from 1 c.c. of serum. Serum 8211.	Serum 16586.
Cherry gum	0·25	0·26	..
	0·5	0·47	..
	0·8	0·48	0·56
	1·6	0·54	..
	2·6	0·41	0·69
	4·0	..	0·67
	8·0	..	0·15
Acacia gum	0·8	0·275	..
	1·6	..	0·34
	2·5	0·285	..
	4·0	0·265	..
	8·0	..	0·32
	12·0	..	0·27
	16·0	Trace	..
Mesquite gum	10	..	0·33
	20	..	0·30
	40	..	0·235
	100	..	0·156

Optimum proportions.—When increasing amounts of gum were added to a series of tubes containing a fixed amount of antiserum, one tube is found to flocculate before those containing more or less gum (optimum proportions with constant antibody, Dean and Webb, 1926). This most rapidly flocculating mixture corresponds approximately to the equilibrium mixture in which, in these cases, traces of gum and of antibody (reacting with the gum in question) could be detected in the supernatant fluid. The antisera formed floccules much more slowly with the gums than with Type II polysaccharide. For example, a mixture of a serum diluted 1/20 flocculated in 13 minutes when mixed with the optimum proportion of Type II polysaccharide; the same serum diluted 1/3 flocculated in 26 minutes when mixed with the optimum proportion of cherry gum.

Effect of precipitation with gums on the amount of antibody reacting with Type II polysaccharide.—The maximal amount of protein nitrogen, precipitable from the antisera by the gums, varied from a little under 1/2 to a little over 1/5 of that precipitable by Type II polysaccharide (Table II). Non-specific protein precipitants may also behave like a specific precipitant for antibody with a zone phenomenon and a most rapidly flocculating mixture; an example has been studied by Dean (1937). Actually a synthetic glucuronic acid compound benzidine-disazo-euxanthic acid prepared by Dr. B. Woolf precipitated protein other than antibody from Type II serum. If the protein

precipitated by a gum is actual antibody protein, the amount of protein in the supernatant fluid, precipitable by Type II polysaccharide, should be correspondingly reduced. This was found to be the case. Gum was added to a given volume of serum ; after standing over night in the cold the precipitate was centrifuged off, washed with cold 0·9 p.c. sodium chloride solution, and its nitrogen content measured. After estimating the optimum proportions of the supernatant fluid with Type II polysaccharide, appropriate amounts of this polysaccharide were added to aliquot parts of the supernatant fluid. This method insured finding the maximum protein precipitable from the supernatant fluid by Type II polysaccharide. The sum of the amount of protein precipitated by the gum and the maximum amount precipitated from the supernatant fluid by Type II polysaccharide was found equal to the maximum amount of protein precipitable from the serum by Type II polysaccharide (Table IV).

TABLE IV.—*Nitrogen Precipitated from Sera by Gums, and from the Resultant Supernatant Fluids by S.S.S. II ; Expressed in mg. of N per* 1 *c.c. of the Original Sera.*

Gum added.	Serum 8211.			Serum 16586.			Serum 16652.		
	A. Nitrogen precipitated by gum.	B. Maximum nitrogen precipitated by S.S.S. II from supernatant.	A + B.	A. Nitrogen precipitated by gum.	B. Nitrogen precipitated by S.S.S. II from supernatant.	A + B.	A. Nitrogen precipitated by gum.	B. Nitrogen precipitated by S.S.S. II from supernatant.	A + B.
None	0	1·29	1·29	0	1·55	1·55	0	0·70	0·70
Cherry gum	0·48	0·82	1·30	0·56	0·98	1·54	..	..	..
Acacia gum	0·28	0·94	1·22	0·34	1·21	1·55	0·19	0·54	0·73
Ghatti gum	0·37	0·90	1·27	..	..	..	..	..	..
Flax mucilage	..	..	..	0·37	1·16	1·53	..	..	..

The optimum proportions of the supernatant fluids with S.S.S. II were also approximately those to be expected, if part of the antibody had been precipitated.

Precipitate formation by the gums, cherry, acacia, ghatti, plum, apricot and possibly flax-seed mucilage with Type II antipneumococcal serum therefore resembles a genuine precipitin reaction. The protein precipitated from the serum by these gums is antibody that reacts with Type II specific polysaccharide.

The supernatant fluids after the addition of one gum to serum were tested with other gums. Except in the case of flax-seed mucilage the results were in agreement with the quantitative estimates of the antibody precipitated. Thus, the supernatant fluid after the addition of the optimum proportion of gum acacia, which formed only a slight precipitate with further gum acacia, formed a definite precipitate on the addition of cherry gum. After the optimal proportions of cherry gum no precipitate formed on the addition of gum acacia. Flax-seed mucilage, however, formed a definite precipitate with the supernatant after the addition of cherry gum, and cherry gum with the supernatant after flax-seed mucilage.

The precipitate formed by gum acacia is not abnormally soluble. The nitrogen precipitated by 2·5 mg. of gum acacia from 1 c.c. of serum 8211 in a total volume of 1·25 c.c. was 0·285 mg. ; when the total volume was 5 c.c. the amount precipitated was 0·275 mg.

None of the gums studied formed more than a very slight precipitate after 24 hours with Type I antipneumococcal serum. The Type III serum at our disposal was weak and formed little precipitate with any of the gums.

The sera used in these experiments were not produced by immunizing with bacteria grown on agar. Cherry, acacia, ghatti, plum, mesquite and apricot gums and flax-seed mucilage did not form precipitates with a serum, supplied by Dr. W. T. J. Morgan, that reacted strongly with agar polysaccharide. The cross reactions under consideration cannot therefore be attributed to an anti-agar antibody in the serum (see Sordelli and Mayer, 1931 ; Morgan, 1936). However, agar polysaccharide, also supplied by Dr. Morgan, formed a precipitate with a strong Type II antipneumococcal serum 16586 but not with the weaker serum 16652.

The reactions of the sera with gum tragacanth and agar polysaccharide were not suitable for quantitative study, as the precipitates were small, gelatinous and not easily separated.

Inhibition.

Precipitate formation by these gums (with the exception of flax-seed mucilage) with Type II antiserum is inhibited by salts* of glucuronic acid (prepared from urine) and of glucuronides. Galacturonic acid does not inhibit in 10 mM. concentration ; mannuronic acid (5 mM.) at most delayed precipitate formation slightly. Lactose, glucose, galactose, saccharic and ascorbic acids have no effect.

Various glucuronides were found to be effective—viz. borneol glucuronide (obtained from urine), the crystalline aldobionic acid of gum acacia (galactose-glucuronide, prepared over the cinchonidine salt), euxanthic acid and hydroxy-anthracene glucuronide (from urine, supplied by Dr. Boyland). Other glucuronic acid derivatives were tested. The benzoyl compound, prepared from dogs' urine by the method of Pryde and Williams (1933*b*) inhibited as well as glucuronic acid. Chondrosin inhibited, but chondroitin sulphuric acid did not. (Both these compounds were kindly supplied by Dr. W. T. J. Morgan.) Methyl-2-4-di-methyl-glucuronide (Pryde and Williams, 1933*a* ; supplied by Dr. Pryde) did not inhibit.

Table V shows the relative efficiency of glucuronic acid and two glucuronides in inhibiting precipitate formation by various polysaccharides with Type II sera. The aldobionic acid from gum arabic appears to be slightly more efficient as an inhibitant than glucuronic acid, and borneol glucuronide slightly less. The amounts of inhibitant required to produce a given degree of inhibition with different gums do not run closely parallel with any of the properties of the gums that we have measured. But, of the vegetable gums studied, cherry gum flocculated most rapidly, formed precipitates in the highest dilution and precipitated most antibody from the antisera ; it was least readily inhibited.

* All reactions are done in approximately neutral solution. When inhibition by an acid is mentioned it is to be understood that a neutral solution of that acid was used.

TABLE V.—*Inhibition by Glucuronic Acid and Glucuronic Acid Compounds.*

Serum 16586.	Glucuronic acid mM. concentration.						Aldobionic acid of gum acacia mM. concentration.						Bornyl glucuroni de mM. concentration.					
	10.	5.	2·5.	1·25.	0·6.	0·3.	10.	5.	2·5.	1·25.	0·6.	0·3.	10.	5.	2·52.	1·25.	0·6.	0·3.
Gum acacia	—	—	—	0	Cl.	Cl.	—	0	0	0	Cl.	—	—	0	0	F.Cl.	Cl.	..
	—	—	—	Cl.	+	++	—	0	0	0	++	—	..	F.Cl.	+	++	++	..
Cherry gum	—	0	F.Cl.	F.Cl.	—	—	—	0	0	F.Cl.	..	..	0	F.Cl.	Cl.	..	..	..
	—	±	±	+	—	—	—	∓	∓	+	..	..	±	+	++	..	..	..
Gum ghatti	—	0	F.Cl.	F.Cl.	F.Cl.	—	—	F.Cl.	F.Cl.	F.Cl.	F.Cl.	—	0	F.Cl.	—	Cl.	..	..
	—	0	∓	∓	±	—	—	0	∓	±	+	—	0	∓	—	±	..	..
Mesquite gum	—	F.Cl.	F.Cl.	F.Cl.	Cl.	—	—	—	F.Cl.	Cl.	+	—	F.Cl.	Cl.	Cl.	+	..	..
	—	Cl.	∓	±	+	—	—	—	+	+	++	—	±	+	++	++	..	..
Plum gum	—	0	0	0	F.Cl.	—	—	0	0	0	+	—	0	0	F.Cl.	F.Cl.	..	..
	—	0	0	±	+	—	—	0	0	+	++	—	±	+	++	++	..	..
Serum 16652.																		
Gum acacia	—	—	—	0	0	F.Cl.	—	—	0	0	F.Cl.	—	—	0	0	0	F.Cl.	..
	—	—	—	0	∓	+	—	—	0	0	+	—	—	0	∓	+	+	..
Cherry gum	—	0	F.Cl.	F.Cl.	..	..	—	0	0	F.Cl.	..	..	—	0	F.Cl.	F.Cl.	..	..
	—	±	±	+	..	..	—	∓	∓	Cl.	..	..	—	+	+	+	..	..

First line, reading after 1 hour at room temperature, second line after night in icebox. F.Cl. = Faint Cloud. Cl. = Cloud.

The reaction of S.S.S. II with dilute serum (*e. g.* 1/10) was delayed by glucuronic acid and derivatives. Subsequent investigations have shown that the amounts of precipitate formed by S.S.S. II with serum diluted 1/3·4 is reduced by 15 mM. glucuronic acid. Precipitate formation by Type I and Type III antipneumococcal sera with the corresponding polysaccharides was not delayed by glucuronic acid.

The acids obtained by hydrolysis of the gums, ghatti, plum and tragacanth inhibited precipitation as effectively as glucuronic acid, when used in the same equivalent concentration, as calculated from the Ba or Ca content of the salts.

A small amount of S.S.S. II was also hydrolyzed by H_2SO_4. The acid fraction inhibited; as far as could be determined, with the small amount available, its efficiency was of the same order as that of glucuronic acid. It delayed, but did not inhibit, precipitate formation by S.S.S. II and antiserum. The neutral fraction did not inhibit.

Cherry gum was added to serum 8211 in optimum proportions. A sample of the supernatant fluid formed only a very slight precipitate on further addition of cherry gum; practically all the antibody reacting with cherry gum had, therefore, been precipitated. The supernatant fluid, diluted 1/8, flocculated with the optimum amount of S.S.S. II in 7 minutes in the absence of glucuronic acid, and in 27 minutes in the presence of 12·5 mM. glucuronic acid.

Precipitate formation by undiluted rabbit antisera to Type II pneumococcus with S.S.S. II was delayed by 12·5 mM. glucuronic acid.

The precipitate formation by Morgan's agar polysaccharide and horse Type II antisera is not inhibited by 10 mM. glucuronic acid.

Attempt to Separate Two Fractions of S.S.S. II.

It might be suggested that the samples of S.S.S. II used were mixtures of two distinct antigens (SA) and (SB), and that the antisera used contained two distinct antibodies (*a*) and (*b*) to correspond; of these, (*a*) only would be precipitated by cherry gum. In the instance of serum 16586, (*a*) would account for 0·69 mg. of antibody N per 1 c.c., and (*b*) for the remaining 0·86 mg. (see Table II).

An attempt was made to separate two such fractions of S.S.S. II by a method comparable to that used by Furth and Landsteiner (1929). 0·24 c.c. of weakly hydrolysed cherry gum (a slight excess) was added to 3 c.c. of serum 16586 in the cold and kept at 0° C. over night. The supernatant fluid obtained should contain only antibody (*b*) as it would no longer give more than a faint trace of precipitate with cherry gum. To 2 c.c. of this supernatant fluid were added 0·165 c.c. of 0·1 p.c. S.S.S. II, about half the equivalent amount. This precipitate should contain only the fraction (SB); it was washed 3 times, dissolved in 0·4 c.c. of N/10 NaOH and heated in a boiling water bath for ½ hour. It was then made slightly acid with HCl and neutralized to phenolphthalein with NaOH. The solution contained recovered polysaccharide, the antibody having been destroyed. Ex hypothesi, this recovered polysaccharide should be (SB) and should precipitate antibody (*b*) only. Optimum proportions of this solution added to serum 16586 precipitated 0·126 mg. of antibody N from 1 c.c. of serum; serial additions of further small amounts precipitated

a further 0·016 mg. of antibody N. Heidelberger and Kendall (1935) have found that such serial additions may precipitate less total antibody N than does a suitable amount of antigen added in one lot. The recovered polysaccharide therefore, in spite of heating with alkali and of serial addition, precipitated nearly all the antibody N from the serum (0·142 mg. out of a total 1·55 mg. per 1 c.c.). This experiment does not support the hypothesis of the presence of two distinct antigens and antibodies.

DISCUSSION.

Cross reactions of the kind here considered may be explained in two ways:

I. It may be supposed that the antiserum contains two qualitatively different types of antibody molecule, which react with different antigenic groups. The antigenic groups may (α) be attached to different molecules, or (β) to the same molecules.

II. It may be supposed that the antibody molecules differ in degree but not in kind, and that some of the molecules react more readily and completely than others with determinant groups differing in composition or arrangement from those of the homologous antigen.

These hypotheses may be illustrated diagrammatically.

Hypothesis.	Substance reacting with antibody.		Antibody involved.
I (α)	Homologous polysaccharides	S_1—A	*a*.
		S_2—B	*b*.
	Heterologous compound	G—A	*a*.
I (β)	Homologous polysaccharide	S<A	*a*.
		S<B	*b*.
	Heterologous compound	G—A	*a*.
II	Homologous polysaccharide	S—A	a_1, a_2, a_3, - - - - -.
	Heterologous compound	G—A′	a_1.

The work of Landsteiner and van der Scheer (1936), in which synthetic antigens were employed, has cleared the outlook considerably. In these experiments it does not seem possible to postulate the presence of two antigenic groups in the immunizing antigens, one of which is shared with the heterologous compounds. So that, for these reactions hypothesis II must be adopted. Precipitate formation with the heterologous compounds is inhibited by lower concentrations of simple haptenes than are required to inhibit precipitate formation with the homologous compounds. Also to inhibit reactions of antiserum with the homologous antigen requires a higher concentration of heterologous than of homologous simple haptene. The amount of a given simple haptene required to inhibit precipitate formation may be considered as a measure of the relative affinities of antibody for antigen and simple haptene. The antibody molecules concerned in these reactions have a greater affinity for the homologous than for the heterologous determinant group.

In the cross reactions considered in this paper hypothesis Iα appears to be disproved by the failure of the attempt to separate the S.S.S. II into two fractions corresponding to two antibodies in the serum; also by the fact that rabbit antisera, which do not precipitate cherry gum, precipitate all the polysaccharide reacting with horse-serum, from preparations of S.S.S. II by the method of Felton, Kauffmann and Stahl (1935).

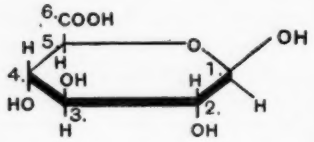
Several objections can be raised to hypothesis Iβ. The group (*a*) would in this instance be glucuronic acid. In the first place this theory would involve the postulation of at least one other determinant group, with appropriate antibody, in order to account for the fact that cherry gum precipitates more antibody than does gum acacia. Both of these, however, are inhibited by glucuronic acid. Again cherry gum should precipitate all the antibody (*a*) from the antiserum, leaving only antibody (*b*). The reaction of this antibody with the corresponding determinant group (B) should not be inhibited by glucuronic acid unless (B) is closely related to (A). Actually serum cleared of the hypothetical (*a*) antibody was found to be delayed by glucuronic acid to the same degree as the untreated serum. Again the reaction with rabbit serum (which does not form precipitates with cherry and acacia gums and therefore would be supposed not to contain antibody (*a*)) with S.S.S. II is delayed by glucuronic acid.

Hypothesis II seems to fit these cross reactions as it fits those studied by Landsteiner and van der Scheer (1936). Precipitate formation by antiserum and the homologous polysaccharide S.S.S. II are considerably less inhibited by glucuronic acid and compounds than are the reactions of the antiserum with the heterologous polysaccharides. The reacting antibodies have therefore a greater affinity for the determinant groups of S.S.S. II than for those of the heterologous polysaccharides. This is borne out by the more rapid flocculation with S.S.S. II, than with, for example, cherry gum. Since glucuronic acid does not completely inhibit precipitate formation with S.S.S. II, it may be supposed that it is a heterologous simple haptene, and that the actual determinant group of S.S.S. II is some related compound. Morgan (1936) has suggested a hypothesis, intermediate between I and II: that the homologous antigen contains antigenic groups (A, B, C, D- - - - -), of which A is dominant, and that the antiserum contains antibodies (*a*), (*ab*), (*abc*), (*ad*), etc., all reacting with the group (A). This hypothesis is distinct from II, when (A) (B), etc., are wholly separate and independent parts of the antigen molecule, but merges into II when A and B form parts of a single small group as in the experiments of Landsteiner and van der Scheer (1936). On Morgan's hypothesis cherry gum might be supposed to have the antigenic groups (A), (B), (C) and gum acacia only (A) and (B); the antibody molecules (*a*) and (*ad*) would not have sufficient affinity for these gums to form precipitates with them.

However, Woolf (in press) finds that synthetic glucuronic acid compounds precipitate as much or more antibody from Type II serum than does cherry gum, although such synthetic antigens can only share one determinant group, the glucuronic acid, with S.S.S. II. The presence of combining groups, other than those for glucuronic acid, in the antibody molecules, would explain the formation of precipitates with flax-seed mucilage, which does not contain glucuronic acid, and the failure of glucuronic acid to inhibit formation of this

precipitate. Also flax-seed mucilage forms a precipitate with serum from which the antibody that reacts with cherry gum has been precipitated.

The failure of inhibition with glucose shows the importance of the acid COOH group, as found also by Chow and Goebel (1935) and Goebel (1936). It is probable that this group combines with the antibody. The combining face of the determinant group is therefore the upper side of the molecule as shown in the structural formula.



Glucuronic acid

This suggestion is supported by the inhibition by chondrosin, which has a large group attached to the No. 4 C atom, but on the underside of the molecule. Also inhibition by the benzoyl compound is consistent with the attachment of the benzoyl group to either the No. 1 or the No. 2 C atom (see Pryde and Williams, 1933*b*; and Quick, 1934). Chondroitin sulphuric acid does not inhibit, possibly owing to the effect of the highly polar groups or to the mutual steric interference of the halves of the molecule. The failure of the compound, in which methyl groups are attached at the No. 2 and No. 4 C atoms is unexpected.

It is remarkable that glucuronic acid is quite as effective, as an inhibitant, as are the glucuronides. This contrasts with the observations with the synthetic glucose antigens (Avery and Goebel, 1929), but Woolf, Marrack and Downie (1935) found that the reactions with synthetic glucuronic acid antigens also were inhibited by glucuronic acid. This suggests a dominant effect of the acid end of the glucuronic molecule.

Although gum tragacanth contains galacturonic acid and not glucuronic acid, the acid obtained by hydrolyzing gum tragacanth (equivalent weight of acid about 300) inhibited approximately as efficiently as glucuronides. It is probable, however, that this preparation contained some partially hydrolyzed gum which may have contributed to the inhibition. Heidelberger (private communication) found that a crystalline preparation of the aldobionic acid of S.S.S. III inhibited precipitate formation by S.S.S. III and Type III antiserum; but that this aldobionic acid, prepared over the salt of an organic base, did not do so.

The uronic acids in the acid nuclei of plum, apricot and ghatti gums have not been identified, but appear not to be glucuronic, galacturonic or mannuronic acids. In view of the inhibition by glucuronic acid it is probable that they are glucuronic acid derivatives; though the startling observations of Goebel and Hotchkiss (1937) make this evidence less convincing than it once appeared.

SUMMARY.

1. Studies have been made of the cross reactions of Type II antipneumococcal sera with various gums.

2. The reactions resemble ordinary precipitin reactions; the gums precipitate varying amounts of antibody from the serum.

3. With one exception the reactions are inhibited specifically by glucuronic

acid and glucuronides ; flocculation of S.S.S. II with Type II antiserum also is delayed.

4. The effect of the introduction of certain substituents in the glucuronic acid molecule has been studied.

5. The bearing of these observations on theories of cross reactions is discussed.

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LIQUID CRYSTALLINE PREPARATIONS OF POTATO VIRUS "X".

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In earlier papers we have presented evidence suggesting that potato virus "X" either contains or is intimately associated with protein. Plants infected with various strains of this virus contain antigens not present in healthy plants, and evidence has been accumulated that these antigens are either the viruses themselves or parts of them (Bawden, 1935; Spooner and Bawden, 1935). Also, potato virus "X" is susceptible to attack by certain proteolytic enzymes (Bawden and Pirie, 1936). Then there was no reason to believe that our purified virus preparations were homogeneous, but now we have isolated the antigens specific to the infected plants in the form of nucleoproteins. Although these nucleoproteins have some properties in common with those isolated from plants infected with strains of tobacco mosaic virus (Bawden and Pirie, 1937*a*), and with cucumber viruses 3 and 4 (Bawden and Pirie, 1937*b*), they differ from them in all respects in which the viruses are known to differ, and the evidence suggests that they are the viruses themselves.

Recently, Stanley and Wyckoff (1937) and Loring and Wyckoff (1937) claim to have isolated a protein of high molecular weight with the properties of potato virus "X" (latent potato mosaic virus) by the high-speed centrifugation of clarified infective sap. As yet, however, they have presented little evidence in support of the claim, for their published results merely show that the sediments contain both virus and protein. No analyses of their products have been given; the statement that their material is a protein being based on the fact that it gives the usual protein colour reactions, and only centrifugal data have been given to support the view that the products are homogeneous.

The potato virus "X" nucleoproteins are much more difficult to obtain in a relatively pure state than are those of the tobacco mosaic virus type. It is necessary to handle the potato virus more carefully, for it is easily destroyed by acids, heating, ageing and proteolytic enzymes. Furthermore, certain physical properties of the potato virus, for example, its higher viscosity and the greater readiness with which it adsorbs impurities, also render its isolation more difficult and more uncertain.

Virus Strains Used.

Salaman (1933) described three strains of potato virus " X ", named S, G and L respectively, and showed that plants infected with one acquire an immunity to further infection with the other strains. The three strains are closely related serologically, possess similar general properties *in vitro* (Spooner and Bawden, 1935 ; Bawden, 1935), and are differentiated because they cause different symptoms in certain host-plants. Chester (1936*b*), using filtered infective sap, showed by means of cross adsorption experiments that each strain contains specific as well as common antigens.

The virulent strain, S, is the only one which produces local lesions, and is therefore the only one with which quantitative infection experiments can conveniently be made. Most of the work described in this paper has been done with this strain. In tobacco (var. White Burley) and in *Nicotiana glutinosa* the S strain produces white necrotic rings at the site of inoculation ; these are followed by systemic symptoms of a ringspot type, all the young actively growing leaves being covered with white, concentric necrotic rings. In tomato (var. Kondine red) it produces an interveinal mottle and black necrotic spots and rings.

When plants are infected with both tobacco mosaic virus and potato virus " X " they show characteristic symptoms, distinct from those produced by either virus alone. Tomatoes infected with both show the well-known experimental or glasshouse streak, and tobaccos show a severe general necrosis, the plants are stunted and the leaves wilt and sometimes collapse.

We have also used the mild, G strain. This produces no recognizable symptoms in infected tomato plants, and its effects on tobacco and *N. glutinosa* are slight. At first the infected plants show no definite symptoms, but after a few weeks they usually develop a faint interveinal mottle.

No significant differences have been found between the nucleoproteins isolated from plants infected with the different strains, except that each produces its characteristic effects when plants are inoculated with it. We have isolated the two virus strains from each of the three hosts, and have found that the nucleoprotein obtained is independent of the species of the host-plant.

Preparation.

Plants are inoculated when small and the leaves picked about a month after infection, that is, with strain S, about three weeks after systemic symptoms appear. The yield of virus per volume of sap is greatest at this time and the isolation is easier, for sap from old plants contains much pigment which is difficult to remove ; also, if old tobacco plants are inoculated with potato virus " X " the infection tends to be localized and the virus does not spread readily out of the inoculated leaves. The leaves are minced and the sap expressed through muslin, heated to 60° C., and then rapidly cooled. This causes a green, flocculent coagulum that is easily thrown down by a few minutes centrifuging at 3000 r.p.m. The heating must be done carefully and with continuous stirring to prevent local overheating, for the virus is rapidly inactivated at temperatures around 66° C.

The brown opalescent supernatant fluid is now either one-quarter saturated

with ammonium sulphate (185 g. per litre) or brought to pH 4·5 by the addition of sulphuric acid. These treatments produce a brown precipitate containing all the virus. After centrifuging, the precipitate from 1 litre of sap is suspended in 100 c.c. of water, neutralized with dilute NaOH, and centrifuged to free from insoluble materials. If the resulting solution is shaken between crossed Nicol prisms it now shows anisotropy of flow. We have been unable to find any materials in healthy plant sap which show anisotropy of flow, and examination of the fluids during the course of preparation in polarized light is the most convenient way of testing for the presence of the virus.

The virus is again precipitated by the addition of from one-third to one-half of a volume of saturated ammonium sulphate solution, centrifuged, and the coloured supernatant fluid discarded. The precipitate is suspended in water and the precipitations with ammonium sulphate repeated until the supernatent fluid is no longer brown. About six repetitions of this treatment are necessary, and a few drops of dilute NaOH solution must be added each time to keep the solution neutral. The precipitates obtained from a given volume of infective sap are smaller with potato virus " X " than they are with strains of tobacco mosaic virus ; they also differ in appearance. The dense white, apparently crystalline precipitates with the intense sheen, so characteristic of tobacco mosaic virus preparations, are not obtained at any stage in the preparations of potato virus " X ". In contrast, these precipitates are light, flocculent and amorphous, and, after centrifugation, form soft, brown, translucent masses. If, however, insufficient ammonium sulphate is added to potato virus " X " preparations to cause precipitation, the fluids do show a sheen, but the sheen is less pronounced than that shown by solutions of tobacco mosaic virus in the same circumstances.

When no further colour can be removed by precipitations with ammonium sulphate, the precipitate is again dissolved at pH 7 and centrifuged until free from insoluble material. The material deposited is dark brown and rich in carbohydrate. It also contains some virus, part of which can generally be recovered in a soluble form after incubation with trypsin. The turbid, brown supernatant fluid is adjusted to about pH 4·5 with acetic acid, and the flocculent precipitate produced is centrifuged down. Generally the whole and always the greater part of the virus precipitates at this pH, but sometimes after prolonged centrifugation at 3,000 r.p.m. the supernatant fluid still shows anisotropy of flow, a part of the virus being soluble at pH 4·5. The virus in solution at pH 4·5 can usually be precipitated by adjusting the pH of the fluid either to 5 or to 4. The acid precipitate is freed from ammonium sulphate by repeatedly suspending in water, centrifuging and discarding the supernatant fluid. As the washings decrease the salt content a larger proportion of the virus becomes soluble at pH 4·5. The actual ratio of the virus soluble to that insoluble at pH 4·5 varies with different preparations, but a part is invariably in each state. No differences have been noticed between the two strains, and both preparations at this stage contain virus soluble and insoluble at pH 4·5. If the preparations at pH 4·5 are frozen solid and allowed to thaw before being centrifuged, most of the virus can be removed at 3000 r.p.m. After this treatment the virus does not dissolve on further washing, and for a given amount of centrifuging it packs more tightly than does the unfrozen

virus. After the precipitate has been washed once or twice, it is suspended in water, frozen solid, thawed, centrifuged, and the washing with water continued. Alternatively, the ammonium sulphate can be removed by dialysis, but this is not to be recommended. When large quantities of virus are handled it is slow, and virus is often lost on an insoluble precipitate which separates during dialysis. The actual loss depends on the quantity of impurities present, and highly purified preparations can be dialysed without losing activity or forming precipitates.

The well-washed, virus-containing, precipitate at pH 4·5 is dissolved by the addition of sufficient N/20 NaOH to raise the pH to 7, and the solution is centrifuged until clear. Tobacco mosaic virus preparations made by precipitation with acid and ammonium sulphate from young infected plants are often colourless, and, if sufficiently concentrated, deposit a liquid crystalline layer. Preparations of the two strains of potato virus " X " made as described from tobacco or *N. glutinosa* plants are clear but brown, and they are never spontaneously birefringent. Preparations from tomato plants are usually less coloured, but otherwise similar. Preparations at this stage are often as infective and as serologically active as those which have been subjected to further fractionations, but these methods are unreliable as tests for purity, for the preparations are still contaminated with sufficient impurities to affect the analytical figures, especially the carbohydrate content, and to prevent the formation of spontaneously birefringent solutions.

Further Purification and the Liquid Crystalline State.

Impure preparations of tobacco mosaic viruses can be fractionated by incubation with trypsin and by sedimenting the viruses in a high-speed centrifuge. Incubation with trypsin is the more satisfactory method, for only in preparations treated thus could no normal plant protein be detected by the sensitive anaphylactic test (Chester, 1936*a* ; Bawden and Pirie, 1937*c*). Both of these methods have been used successfully in the further purification of potato virus " X ".

We have published data (Bawden and Pirie, 1936) on the rate at which trypsin inactivates potato virus " X ". We now find that the activity of partially purified preparations, containing about 0·4 p.c. of solids, is reduced by one-third by incubation with 0·05 p.c. pancreatin (Merk) for 90 minutes at 38° C. and pH 7·4. During incubation there is a loss of anisotropy of flow corresponding to the fall in activity. After six hours in these conditions the preparations show no anisotropy of flow, and the activity is reduced to one-eighth. Before testing the infectivity of the incubated mixtures, the virus must be freed from trypsin by precipitations with ammonium sulphate and acid, for the presence of trypsin in the inoculum greatly reduces the infectivity.

The coloured virus preparations containing about 0·4 p.c. of solids are incubated with 0·05 p.c. pancreatin for only 90 minutes in the conditions stated. Brown material can now be removed by centrifuging, and the virus is precipitated by the addition of one-third of a volume of saturated ammonium sulphate solution. The precipitation is repeated until the supernatant fluid is no longer coloured.

Although the virus is not precipitated until the concentration of ammonium sulphate is greater than one-sixth of saturation, the precipitated virus will not dissolve until the concentration of ammonium sulphate is reduced to about one-twentieth of saturation. One incubation with trypsin is usually sufficient to give colourless preparations, but sometimes, especially with material from older plants, it is necessary to repeat the treatment. The ammonium sulphate is removed by precipitating the virus at pH 4·5, and washing with water. The virus is dissolved in sufficient N/10 NaOH to bring the pH to 7. Before the last centrifugation at pH 4·5 the preparation should be frozen and thawed, otherwise the neutral solution will be too dilute to give a liquid crystalline layer. Even after incubation with trypsin it is difficult to obtain clear, homogeneous preparations of potato virus " X " without using a high-speed centrifuge. With experience it is possible to obtain colourless solutions, which, if more concentrated than about 3 p.c., will separate on standing into two layers. The lower layer is liquid crystalline, has the greater solid content and is clear, while the upper layer is turbid and shows anisotropy of flow.

By the use of trypsin it is possible to purify the coloured virus preparations without losing more than one-third of the virus. High-speed centrifugation is a quicker and more certain method of purification, but less of the virus is recovered in the purified state. When the partially purified preparations, containing from 1 to 2 p.c. of solids, are centrifuged for three hours at 14,000 r.p.m. (16,000 times gravity), from a fifth to a third of the virus is sedimented in the form of turbid birefringent pellets, containing from 15–20 p.c. of solids. When dissolved in from 5 to 10 volumes of water, the pellets give opalescent solutions, from which brown precipitates, rich in carbohydrate, can be removed by light centrifugation. The remaining supernatant fluid is now almost colourless and is spontaneously birefringent. If the solutions are now diluted until they are no longer spontaneously birefringent, and again centrifuged for three hours at 16,000 times gravity, one-half or more of the virus sediments. The birefringent pellets again contain small amounts of insoluble material, but are now colourless. The insoluble material is removed by dissolving the pellets in water, and lightly centrifuging. More virus can be recovered from the supernatant fluids from the high-speed centrifugations either by a further period of centrifugation, or by the use of trypsin.

Prepared in this manner 2 p.c. solutions of both strains of the virus are colourless and only faintly opalescent. At room temperature they are liquid crystalline, and they may keep this property until diluted to about 1·5 p.c. The intensity of anisotropy of flow shown by dilute solutions is of the same order as that shown by solutions of tobacco mosaic virus. Solutions of potato virus " X " are more viscous, and the separation of originally homogeneous solutions into layers takes place more slowly. With a 2·5 p.c. solution some separation is noticeable after a day at 0° C., but the interface between the layers is not sharp until the solution has stood undisturbed for a week. With tobacco mosaic virus the layering phenomenon could be used in the further purification, but with virus " X " it cannot, for we have been unable to get a preparation of this virus to settle into layers until the purification has been carried so far that the differences in properties and concentration of the materials in the two layers are extremely small.

More care must be taken over the storage of solutions of virus " X " than of tobacco mosaic virus, for they are more susceptible to contamination with bacteria, and they lose their birefringence fairly rapidly at room temperature. Preparations of virus " X " kept for some weeks decompose, even when stored in the cold, and become viscous. A 0·5 p.c. solution of old virus may be too gelatinous to flow at 0° C. ; in this condition the preparations are sometimes feebly birefringent, and they are highly thixotropic. When shaken or warmed they will again flow and then show anisotropy of flow, but this is less intense than that shown by a solution of freshly prepared virus of the same concentration.

Although the optical properties of neutral solutions of freshly prepared potato virus " X " and those of tobacco mosaic virus are similar, they are distinct when the viruses are precipitated with acid or with ammonium sulphate. Potato virus " X " precipitates from highly purified preparations are amorphous and difficult to see, although they are thrown down by centrifuging at 3000 r.p.m., and they are detected by the increased readiness of the fluid to hold bubbles. The anisotropy of flow shown by tobacco mosaic virus preparations is greatly increased by precipitating the virus by either of the methods ; neutral solutions of tobacco mosaic virus diluted beyond the point at which they show anisotropy of flow will show the phenomenon if the virus is precipitated with either acid or with ammonium sulphate. In contrast, neutral solutions of potato virus " X ", at concentrations at which they show definite anisotropy of flow, lose this property when the virus is completely precipitated with acid or ammonium sulphate. When acid or ammonium sulphate is added to solutions of potato virus " X " in quantities insufficient to cause precipitation, their behaviour more nearly resembles that of tobacco mosaic virus solutions. At about pH 5, or in about 10 p.c. saturated ammonium sulphate, the potato virus preparations develop a slight sheen and show increased anisotropy of flow.

We have shown that the tobacco mosaic type viruses give paracrystalline precipitates with clupein, resembling those obtained with acid or ammonium sulphate. When clupein is added to neutral solutions of potato virus " X ", precipitates are formed, which also resemble those produced by the addition of acid or ammonium sulphate. The precipitates are amorphous and difficult to see, but are readily separated by centrifuging at 3000 r.p.m. Thus, although we have obtained potato virus " X " in the form of birefringent solutions and birefringent jellies, closely resembling in optical properties those of tobacco mosaic virus, we have been unable to obtain any solid preparations of this virus which could be described as " crystals ". Similarly, there is a difference in the kinds of inclusion bodies formed by the two viruses, for whereas plants infected with tobacco mosaic virus contain both amorphous " X "-bodies and hexagonal crystals, plants infected with virus " X " contain only amorphous " X "-bodies.

Analyses.

The physical properties of potato virus " X " are greatly affected by processes of purification which have no measurable effect on the activity or the analytical figures. The effects with tobacco mosaic virus were much

smaller, and more confident statements could be made about properties. The properties of potato virus " X " described in this paper are those characteristic of preparations believed to be the purest, but it is impossible to make positive statements until some better criteria of purity are available.

Landsteiner and Heidelberger (1923) and Northrop (1930) have pointed out the importance of solubility measurements for determining the purity of protein preparations, and they suggest that when the solubility is independent of the amount of solid protein present it is probable that the preparation is pure. We have been unable to make similar measurements on the solubilities of any of the viruses we have used. No solid phase separates from solutions as they are concentrated, but the fluids become more viscous and turn to gels. If experiments are carried out in conditions in which a solid phase will separate, *e.g.* at pH values or at ammonium sulphate concentrations insufficient for complete precipitation of the viruses, the amount remaining in the supernatant fluid (*i.e.* the solubility) depends upon the intensity of centrifugation.

As the maximum solid content of solutions showing only anisotropy of flow (top layer) depends upon the purity, the formation of a liquid crystalline layer is valuable as an indication of purity. When the two layers are in equilibrium, the difference in concentration between them becomes less as the purity increases. Therefore it is possible that with a perfectly pure virus preparation the two layers could not occur together, for a small addition of water or of virus, or a rise or fall in temperature, would change the whole system from one state to the other.

Preparations of potato virus " X " with reasonably constant carbon, hydrogen, nitrogen and ash contents, are easily made by precipitation methods, for the probable impurities have compositions similar to the virus. The carbohydrate content of a preparation, measured by an orcinol method (Pirie, 1936), is a more reliable index of the purity. The coloured, impure preparations described in this paper have carbohydrate contents of from 5 p.c. to 10 p.c. When these preparations are centrifuged at 14,000 r.p.m., the pellet of virus contains brownish flakes, which do not dissolve when the pellet is suspended in water. A second centrifugation of the redissolved virus, however, usually gives more of these insoluble flakes. Analysis on the supernatant fluids from the high-speed centrifugation, on the insoluble flakes, and on the soluble birefringent sediment, show that the first two are rich in carbohydrate and that the last is not. By repeating the process of centrifugal fractionation a few times, preparations can be obtained with a carbohydrate content of from 2·5 p.c. to 3 p.c. The extra carbohydrate is not an essential constituent of the virus, for reducing the carbohydrate content to this level does not decrease the activity of the virus preparation. We have been unable to reduce the carbohydrate content to below 2·5 p.c. by centrifugation. Removal of this carbohydrate results in inactivation of the virus.

It is well known that proteins readily adsorb other materials and this presumably accounts for the sedimentation of pigmented material, rich in carbohydrate, together with the virus during high-speed centrifugation, and for the fact that the impurity is only gradually removed by repeated centrifugation. Because of this property of proteins it is impossible to assume that material sedimenting in intense gravitational fields is necessarily all virus, for

unless there are other reasons for considering the material to be homogeneous, the sedimentation rate measured may merely be that of an adsorption complex containing virus.

Highly purified, liquid crystalline preparations of potato virus " X " have given the following analyses, and no differences have been detected between the analytical figures of the two strains used.

Carbon . . .	47·7 to 49·5 p.c.
Hydrogen . .	7·1 ,, 7·7 ,,
Nitrogen . .	15·7 ,, 17·0 ,,
Phosphorus . .	0·4 ,, 0·5 ,,
Carbohydrate . .	2·5 ,, 3·0 ,,
Ash . . .	2·0 ,, 2·5 ,,

Within the limits of experimental error, therefore, virus " X " has the same analytical composition as the strains of tobacco mosaic virus and cucumber viruses we have already described. As a nucleic acid can be isolated from inactivated preparations of potato virus " X ", it seems that this, like the other viruses, is also a nucleoprotein.

Activity.

The yields of potato virus " X " obtained have averaged about 100 mg. per litre of infective tobacco sap, but they have varied with the number and type of treatments used to purify the sample. The final yield represents only a part of the virus present in infective sap, for the treatments used destroy much of the virus ; from the serological titres of clarified, infective saps it seems probable that the virus content can be as great as 500 mg. per litre.

The purified virus preparations were tested for their infectivity and their serological activity. Infectivity tests with the S strain were made by the local lesion method, the hosts used being tobacco and *N. glutinosa*. Tobacco proved much the more sensitive, and virus solutions giving some hundreds of lesions on a tobacco leaf often gave only a few on a leaf of *N. glutinosa*. The first-formed true leaves are most sensitive, the young upper leaves giving few lesions. For this reason the first, second and third leaves were used, and 1 c.c. of a solution containing a known weight of the purified virus preparations was carefully rubbed over the surfaces of six leaves. Bald (1937) has shown that the presence of electrolytes reduces the numbers of lesions produced by potato virus " X ", and the dilutions were therefore made in water.

Serological titres were measured by the methods previously described. Antisera were prepared both by a series of intraperitoneal injections of crude infective sap, and by intravenous injections of the purified viruses. The titres of the virus preparations were found to be independent of the method of preparing the antiserum, and to be the same whether the individual strains were titrated against their homologous or their heterologous antisera. Changes in the antiserum affected only the optimal precipitating points of virus preparations and not their precipitating end-points.

The results of infectivity and serological tests with six purified preparations of strain S are given in Table I. The infectivity of preparations of strain G was estimated by inoculating plants with 1 c.c. of solutions containing known

amounts of protein, and finding the smallest amount required to produce systemic infection. Inoculated plants which showed no symptoms were reinoculated with the S strain. If susceptible to infection with the severe strain they were taken as uninfected. The amount of protein required to produce systemic infection varied in different tests from 10^{-7} to 10^{-9} g. The serological titres of the purified preparations of the G strain have also varied from $\frac{1}{3 \times 10^6}$ to $\frac{1}{6 \times 10^6}$. The higher serological titres have usually been obtained with preparations from plants grown in the summer and the lower from plants grown in the winter.

TABLE I.—*Activity of Purified Preparations of the S Strain of Potato Virus "X".*

Serological titre.	Infectivity on tobacco (var. White Burley). Average number of lesions per leaf at various dilutions.					
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
$\frac{1}{4 \times 10^6}$	775	214	35	3	0·6	0
$\frac{1}{6 \times 10^6}$	550	160	40	6	2	0·3
$\frac{1}{4 \times 10^6}$	95	15	2	0·3	0	0
$\frac{1}{3 \times 10^6}$	450	252	31	6	1	0·3
$\frac{1}{6 \times 10^6}$	196	38	9	2	0·3	0
$\frac{1}{5 \times 10^6}$	660	220	42	7	1	0

The serological titre is expressed as the highest dilution, in grams per c.c., at which there was a visible precipitate after 24 hours when 1 c.c. of virus solution was mixed with 1 c.c. of antiserum at 1 in 50. The dilutions in the infectivity tests are given as grams per c.c. Six leaves were rubbed with 1 c.c. The tests were made at different times with different preparations, and the large differences in the infectivities are due to differences in the susceptibilities of the host-plants. The results of serological tests are constant and reproducible, but the results of infectivity tests vary greatly with the host-plants.

Potato virus "X" does not precipitate with tobacco mosaic virus antiserum, and all our purified preparations were tested against this antiserum to ensure that they were not contaminated with tobacco mosaic virus. This was necessary for, although the two viruses have such different physical properties, none of the purification methods described in this paper will separate them completely one from the other. As tobacco mosaic virus is more stable, pure preparations of this can easily be obtained from plants infected with both viruses by exposing the extracted sap to conditions which inactivate potato virus "X" but not tobacco mosaic virus, but as yet it has proved impossible to isolate from such a mixture a preparation of potato virus "X" free from tobacco mosaic virus. All the plants used in this work were also carefully examined for the symptoms characteristic of a mixed infection.

The decrease in numbers of local lesions accompanying dilution is not directly proportional to the dilution, but more are obtained at high dilutions than would be expected. With tobacco mosaic virus this deviation from the expected is much more pronounced, and sometimes dilution may actually result in an increased number of lesions. Bald and Briggs (1937), from a mathematical interpretation of the dilution curve of purified preparations of tobacco mosaic virus, state that the deviation from the expected agrees well with the suggestion that the virus particles are aggregated in concentrated solutions, and that dilution breaks up the aggregates. Although this explanation may account for the behaviour of purified preparations when diluted, it is unlikely that it can account for the deviations in all dilution curves. For example, the dilution curve of crude tobacco mosaic sap resembles that of purified virus preparations. As we have shown that aggregation into rods is largely an effect of purification, it is improbable that the results obtained by diluting crude sap can also be explained solely by the breaking up of the virus aggregates. It is perhaps more probable that the deviations in the dilution curve of crude sap are caused by relatively inactive complexes of virus-host material breaking up when diluted. Some support for this comes from the fact that the addition of healthy tobacco sap to infective sap causes a reduction in the infectivity, but when the mixtures are highly diluted with water they regain most of their activity.

As solutions of the purified virus " X " show anisotropy of flow, and in some conditions show a sheen, the particles in them are presumably rod-shaped. These rods also appear to be formed by the linear aggregation of smaller particles. The fact that the fall in infectivity with dilution is more proportional to the dilution with purified preparations of potato virus " X ", suggests that the aggregated rods of this virus are more stable than those of tobacco mosaic virus, or, alternatively, if they do break up on dilution, that they are built up of fewer components.

Evidence that small particles of virus " X " aggregate to form rods when precipitated, comes from experiments comparing the filterability, activity and optical properties of crude infective sap with those of the purified preparations. These experiments are similar in type to those described in more detail with tobacco mosaic virus (Bawden and Pirie, 1937*a*). Smith and Doncaster (1936) found that potato virus " X " in untreated, clarified sap passed through collodion membranes with an average pore size of 150 mμ., but after partial purification it would not pass through a membrane with an average pore size of 240 mμ., and they suggested that the virus aggregated. Filtration experiments with out purified preparations have given similar results ; the virus in untreated sap has passed through membranes with an average pore size of 150 mμ., but with solutions of the purified virus we have been unable to obtain infectious filtrates through membranes with an average pore size of 400 mμ. The virus need not be carefully purified to show this reduction in filterability, for after one precipitation with acid or ammonium sulphate it will no longer pass through a membrane of pore size 150 mμ.

Similarly treated potato virus " X " preparations with the same serological activity are equally infective, but preparations treated differently can have the same serological activity but different infectivities (Bawden, 1935). Crude

sap always produces more lesions than a solution of the purified virus with the same serological titre. Any type of aggregation of the virus particles would result in a reduction of the filterability, and in a reduction of the ratio of infectivity to serological activity. We suggest that the particles aggregate to form rods because of the optical properties of the purified preparations, for only an aggregation which results in a definite departure from the spherical shape could increase the degree of anisotropy of flow shown by a given amount of virus. Direct comparisons of the optical properties of the virus before and after treatment cannot often be made, because the virus content of clarified sap is too small for anisotropy of flow to be seen. After precipitation with ammonium sulphate and resolution in a volume of water equal to that of the original sap, a few samples have shown anisotropy of flow, and when dissolved in a tenth of a volume of water they all show the phenomenon.

Inactivation of Potato Virus "X".

We have shown previously that potato virus "X" preparations can be inactivated in two ways. Some treatments cause loss of both serological activity and infectivity, whereas others, *e.g.* nitrous acid and formaldehyde, destroy infectivity without in any way affecting the serological activity (Bawden 1935; Bawden and Pirie, 1936; Bawden, Pirie and Spooner, 1936). With tobacco mosaic virus similar results were obtained, and treatments of the second type were found to have no effect on the ability of the virus preparations to show anisotropy of flow and to form liquid crystalline solutions (Bawden and Pirie, 1937*a*). With the purified preparations of virus "X" we now find that treatments denaturing the protein cause loss of infectivity, serological activity, and the ability to show anisotropy of flow, whereas treatments causing loss of infectivity without denaturing the protein leave the serological activity and the ability to show anisotropy of flow unaffected.

Heating.—Purified preparations of tobacco mosaic virus are unaffected by long periods of heating at 70° C., but when heated to above 90° C. they break down to give an insoluble coagulated protein and a soluble free nucleic acid. Potato virus "X" is much more sensitive to heating, and the effect of heat seems to be different. Heating neutral salt-free solutions for some time at 60° C. has no apparent effect on their activity or on their ability to show anisotropy of flow. A few minutes at temperatures above 66° C. is sufficient to inactivate; the solutions become more opalescent, and lose their infectivity, serological activity and anisotropy of flow, but no precipitate is formed. Further heating has no apparent effect, and such solutions can be boiled without the separation of a protein coagulum. The addition of a little neutral salt to the boiled solution causes the precipitation of some denatured protein, which is free from phosphorus. If the fluid is now acidified gradually, a succession of protein fractions is obtained, and the precipitation of protein is complete when the pH of the fluid is about 4. These fractions contain phosphorus, the amount increasing with the increase in acidity required to cause precipitation. The addition of a strong acid to the remaining supernatant fluid causes the separation of a gummy precipitate of crude nucleic acid, apparently similar to that isolated from inactivated tobacco mosaic virus.

If the virus preparation used for such an experiment has a carbohydrate content of 3·5 p.c. or higher, the first precipitates formed are rich in carbohydrate, although they may contain less than 0·1 p.c. of phorphorus. By incubating this precipitated protein with trypsin a brownish precipitate can be made ; this contains a pentosan similar to that which can be removed from the preparation by repeated high-speed centrifugation, and it accounts for most of the carbohydrate of the first denatured protein. Good preparations of the virus, containing only 2·5 p.c. of carbohydrate, give no precipitate of this type, and this is confirmatory evidence for the view that virus preparations containing more than this amount of carbohydrate can be still further purified.

Drying.—If leaves, or expressed sap, from plants infected with potato virus " X " are completely dried they lose their infectivity (Smith, 1933). Highly purified preparations of the virus can be dried and still be active, but the activity and physical state of the dried virus are so greatly affected by the salt content of the solution, and by the method used for drying, that reproducible results are not easily obtained. Alternate freezing and thawing has no effect on the virus solutions, and the virus solutions were dried frozen in a vacuum desiccator.

With incomplete drying, that is, using sodium sulphate or ammonium sulphate as the desiccating agent, the virus film is birefringent, and when taken up in water shows little loss of activity. When completely dried over phosphorus pentoxide, the virus film is not birefringent, and on resolution there is always reduction in the infectivity and serological activity. The loss depends on the pH value of the solutions dried : in pH 6 buffer the activity is reduced to about one half of the original, while at pH 4 and pH 8 more than 90 p.c. of the activity may be lost, and much of the material is rendered insoluble. After complete drying at from pH 6 to pH 7, about one-quarter of the material is not soluble in water, and the soluble portion has lost to a considerable extent its ability to show anisotropy of flow. The soluble part can be fractionated by both high-speed centrifugation and by incubation with trypsin, and fully active virus recovered showing the usual amount of anisotropy of flow. It seems, therefore, that the disintegration products produced by drying potato virus " X ", like those from tobacco mosaic virus, impede the arrangement of the particles that is necessary for anisotropy of flow to be seen. The insoluble part of the dried virus has a slightly higher nucleic acid content than the original virus, and when extracted with 80 p.c. acetic acid it readily gives a residue of crude nucleic acid.

Acid.—The virus precipitates optimally from pure preparation at about pH 4·5, but it can be completely sedimented by long periods of centrifugation at low speeds over a considerable range on both sides of this point. At room temperature and pH 4 denaturation of the protein is slow, but at pH 3 a few hours are sufficient to denature and inactivate.

When treated with 80 p.c. acetic acid, both strains of potato virus " X " break down to give proteins and nucleic acid, but it is more difficult to separate the two components than it is with similarly treated viruses of the tobacco mosaic type. The most promising results have been obtained by adding four volumes of acetic acid to the tightly-packed precipitates formed by centrifuging the virus preparations at pH 4·5. The proteins rapidly dissolve and leave

a residue of crude nucleic acid, or nuclein, containing about 4 p.c. of phosphorus. The nucleic acids obtained from the strains of virus " X " have not yet been investigated in any detail.

Alcohol.—The addition of an equal volume of 80 p.c. alcohol to infective sap precipitates virus " X " completely, and the sediment must be washed several times in water before all the virus will go into solution. In the purified salt-free preparations, precipitates form only slowly in 50 p.c. alcohol; with 60 p.c. or more alcohol the precipitation is rapid, and the sedimented virus readily redissolves in water. Denaturation does not proceed at all rapidly until the concentration of alcohol reaches 85 p.c. In the presence of calcium chloride the purified virus is immediately precipitated by 40 p.c. alcohol, and the resulting sediment is insoluble in water. All the virus, however, is not inactivated, for some lesions are obtained when plants are rubbed with the insoluble precipitate. Janssen (1937) has described a similar effect with the virus causing foot-and-mouth disease of cattle.

Sodium dodecyl sulphate.—Pancreas contains, in addition to trypsin, a substance soluble in petrol ether which inactivates potato virus " X " (Bawden and Pirie, 1936). This substance also inactivates some animal viruses (A. Pirie, 1935), and the type of inactivation is presumably analogous to that obtained with soap, bile and other related surface-active substances, which are well known as inactivators of certain viruses. A. Pirie (1938) has also studied the inactivation of several animal viruses by surface-active substances used as commercial wetting and spreading agents (long chain sulphonic acids, and the sulphuric half esters of long chain alcohols). We find that these agents rapidly inactivate potato virus " X ", and of those that we have tried sodium dodecyl sulphate is the most effective.

TABLE II.—*Effect of Incubating* 1 *p.c. Solutions of Potato Virus "X" with* 0·33 *p.c. Sodium Dodecyl Sulphate.*

Time of incubation.	Serological titre.	Infectivity. Average number of lesions per leaf. Inoculum virus content 10^{-4} gm. per c.c.
Mixed, but not incubated	$\frac{1}{2 \times 10^6}$	420
40 mins.	$\frac{1}{10^5}$	45
150 mins.	$\frac{1}{10^4}$	3·6
300 mins.	No. precipitate at $\frac{1}{2 \times 10^3}$	0

Table II shows the results of one inactivation experiment. 1 c.c. of fluid containing 10 mgm. of purified virus and 3·3 mgm. of sodium dodecyl sulphate was incubated at 38° C. At different intervals 0·2 c.c. of the mixture were removed and immediately diluted with 1·8 c.c. of water. Dilution to this extent almost stops the action of the wetting agent, for the virus is but little

affected by 0·03 p.c. sodium dodecyl sulphate, although the more concentrated virus solution is rapidly inactivated by 0·3 p.c. The inactivated virus shows no anisotropy of flow, and the anisotropy of flow diminishes during incubation at approximately the same rate as the activity. Nothing can be sedimented from the completely inactivated virus preparations by centrifuging for 3 hours at 16,000 times gravity, but the protein can still be precipitated with acid or with ammonium sulphate.

Irradiation.—Solutions of potato virus " X " exposed to either X-rays or to ultra-violet light lose their infectivity, the loss being proportional to the time of irradiation. The inactivated preparations are unchanged in appearance, and they still show anisotropy of flow and react specifically with virus anti-serum.

TABLE III.—*The Effect of X-rays and Ultra-violet Radiation on Solutions of Potato Virus "X".*

Radiation.	Time.	Serological titre.	Infectivity. Average number of lesions per leaf. Concentration of inoculum 10^{-4} gm. per c.c.
X-rays	100 mins.	$\frac{1}{2 \times 10^6}$	22
	360 ,,	$\frac{1}{2 \times 10^6}$	0
Ultra-violet	3·3 mins.	$\frac{1}{3 \times 10^6}$	40
	10 mins.	$\frac{1}{3 \times 10^6}$	1
	30 ,,	$\frac{1}{2 \times 10^6}$	0
Control	Not irradiated	$\frac{1}{3 \times 10^6}$	342

The results of one experiment are given in Table III. 0·2 p.c. solutions of purified virus were used. In the ultra-violet test a layer 1 mm. thick, under a quartz plate, was irradiated at a distance of 8 cm. from a 2 amp. lamp. In the X-ray test, the solution was irradiated at a distance of 8 cm. from a copper anticathode of an X-ray tube run at 30 k.v. and 20 m.a.

Hydrogen peroxide.—Stanley (1936) has shown that hydrogen peroxide inactivates tobacco mosaic virus without affecting its specific reaction with antiserum. We have confirmed this, and also obtained similar results with potato virus " X ". Virus " X ", however, is more sensitive to oxidation, and the treatment must be carried out more cautiously than with tobacco mosaic virus. Treatment with concentrated H_2O_2 denatures the protein, and causes loss of all characteristic properties, while treatment with dilute solutions destroys infectivity without affecting the serological reactions or the anisotropy of flow. At room temperature, solutions of potato virus " X " are rapidly inactivated by H_2O_2 at concentrations between 0·2 p.c. and 1 p.c. without the serological activity being affected, but concentrations above 1 p.c. denature the virus.

DISCUSSION.

The isolation of tobacco mosaic virus as a nucleoprotein was little reason for thinking that other viruses were necessarily chemically similar, for it is an exceptionally stable virus and its properties differ greatly from those of other viruses. Potato virus " X " is more stable *in vitro* than many viruses, but its properties are more typical of the virus group as a whole, and the recognition of this virus as a nucleoprotein does suggest that other viruses may also be found to be proteins. It is interesting to note that nucleoprotein has been found in all the purified preparations of viruses and bacteriophages that have been tested for it, and in this respect the viruses seem to be similar to all known cells. The plant viruses which we have purified are apparently solid masses of nucleoprotein, for the preparations contain no appreciable amounts of water or of diffusible substances, and the X-ray measurements show that the virus particles have a perfect internal regularity of the type found in some large protein molecules. Recent work on coli phage (Schlesinger, 1936) and staphylococcus phage (Northrop, 1937) suggests that these are also nucleoproteins. It is perhaps improbable that this will be found to be a general feature of viruses, and it is more likely that some viruses, especially the larger ones, will be found to be more highly organized and to contain substances other than nucleoprotein. With vaccinia elementary bodies there is already some evidence in support of this view (Hughes, Parker and Rivers, 1935; Salaman, 1937).

In spite of the chemical relationship which exists between the known purified viruses, there are considerable differences in their properties. For example, potato virus " X " and tobacco mosaic virus break down differently when heated, and the former is susceptible to tryptic digestion while the latter is not. Also, nothing analogous to the linear aggregation, so characteristic of the purified preparations of these two virus types, has been noticed with the animal viruses or phages, but that these may be affected by purification processes is suggested by the observation of Schlesinger and Galloway (1937) that foot-and-mouth virus is more readily adsorbed on surfaces when partially purified than it is when in the crude state. The power of linear aggregation, however, does not seem to be a general property of plant viruses, for we have been unable to obtain anisotropic preparations of some plant viruses, although highly active, protein-containing sediments can readily be obtained by high-speed centrifugation.

The method by which these viruses increase in the infected plants is unknown, but the fact that the presence of strain G prevents the multiplication of strain S suggests that the two behave identically. Healthy plants do not seem to contain any proteins similar to those isolated from the infected plants. Healthy plant sap heated to 60° C. gives a coagulum similar to that obtained by heating infective sap, but a smaller precipitate separates when the remaining supernatant fluid is one-quarter saturated with ammonium sulphate. This precipitated material shows no anisotropy of flow when redissolved; it is also less stable than that obtained from infected plants, and the quantity becomes much reduced by repeated precipitations with ammonium sulphate. Nothing can be sedimented from solutions by 3 hours centrifugation at 16,000 times gravity.

Although the 2 strains used cause such different symptoms, we have found no significant differences between them, either in the yields obtained or in the physical or chemical properties of the isolates. No doubt there are small differences of the type found between the various strains of tobacco mosaic virus, and Chester's (1936*b*) experiments show that the strains possess specific as well as common antigens. As plants infected with the different strains do not contain greatly different amounts of virus, it is improbable that the symptoms are a direct result of virus multiplication in weakening the host. It is more probable that the symptoms are the result of a secondary effect of the virus on the host, produced by active side groups, and that the different strains cause their characteristic symptoms because they possess specific side groups. The fact that each strain contains specific antigens is some support for the view that they possess such specific groups.

SUMMARY.

Methods are described for the isolation of nucleoproteins from *N. tabacum*, *N. glutinosa* and *Lycopersicum esculentum*, infected with the S and G strains of potato virus " X ". These have not been isolated from healthy plants, and the available evidence suggests that they are the viruses themselves. Infections were obtained with 10^{-9} gm., and specific serological reactions with $\frac{1}{6 \times 10^6}$ gm. Concentrated solutions are spontaneously bi-refringent and dilute solutions show anisotropy of flow; when sedimented by high-speed centrifugation the nucleoproteins form birefringent jellies, but when precipitated with acid or ammonium sulphate the material appears amorphous under the microscope.

The filterability of the virus after purification is less than that of the virus in untreated sap, and purification appears to cause the virus particles to aggregate into rods.

Two types of inactivation are described: one leads to a loss of infectivity without changing the optical properties or serological reactions, whereas the other denatures the protein and destroys all three. The effects of heating, drying, acid, alcohol, sodium dodecyl sulphate, irradiation with X-rays and ultra-violet light, and hydrogen peroxide are described. The properties of virus " X " are compared with those of tobacco mosaic virus, and the results discussed.

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THE ISOLATION OF ANTIGENIC SUBSTANCES FROM STRAINS OF *BACT. TYPHOSUM*.

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IN a recent paper one of us (W. T. J. M., 1937) described a method for the isolation of a specific antigenic substance from *Bact. dysenteriæ* (Shiga). This substance is apparently free from protein and as far as it has been determined, it induces in the rabbit an antibacterial response indistinguishable from that produced by the untreated bacterial cells.

We have extended the use of this method to the study of *Bact. typhosum*; this communication deals with the extraction of two of its antigenic components and with some of their immunological and chemical properties.

Methods.

The strains of Bact. typhosum *examined.*—These are representative of the O agglutinable or O forms, the O inagglutinable or O + Vi forms and the so-called rough Vi forms believed to contain only Vi antigen; they have been described by Felix and Pitt (1934*a* and *b*, 1935). We are indebted to our colleague Dr. Felix for these strains and also for valuable information concerning their maintenance.

The maintenance of the cultures.—The strains were kept at 37° C. on a trypsin-digest agar; they were passaged daily and plated on this medium at least once a week. The antigenic condition of each strain was regularly

tested by agglutination with pure O, pure Vi and pure H sera which had previously been selected as laboratory " standards ". This method, which is used by Felix, detects the occurrence of any slight variation in the antigenic content of the strains.

The production of mass cultures.—A strain to be grown for the extraction of antigens was " seeded " on trypsin-digest agar contained in enamelled trays measuring 29 × 43 cm. Usually 36 of these trays comprised a batch. The growth obtained after incubation for 18 hours at 37° C. was removed with a glass spatula, suspended in cold physiological saline and centrifuged. The supernatant fluid was then carefully siphoned off and the bacterial deposit vigorously shaken with three or four times its volume of ice-cold acetone. The organisms were again deposited by centrifuging, washed twice in a similar manner with cold acetone and finally dried *in vacuo* or at 37° C. As soon as the acetone had been removed the dry bacterial powder was weighed and stored over phosphorus pentoxide at −10° C. After some experience it was found that the growth obtained from 36 trays could be harvested and treated as described within a period of 3 or 4 hours, the yield of dry organisms varying with the strain and amounting to approximately 0·5 gm.–1·5 gm. per tray.

The extraction of the antigenic substances.—The antigenic substances that we have isolated have been prepared from the dry bacteria by extraction with diethylene-glycol according to the method previously described (Morgan, 1937). The extracted substances have generally been further freed from non-specific material by fractionation from ice-cold aqueous solution with acetone or alcohol that has previously been cooled to −10° C. In experiments with *Bact. dysenteriæ* (Shiga) it was found that 0·5 to 1·0 p.c. solutions of the extracted material were not precipitated from aqueous solution by acetone until this reagent reached a concentration of 50 p.c. The extracts of O, O + Vi and rough Vi strains of *Bact. typhosum*, however, commenced to precipitate from solution at about 33 p.c. by volume of acetone and precipitation was practically complete when an equal volume—approximately 50 p.c.—of this reagent was added. Similarly, alcohol was found to precipitate the antigenic complex from *Bact. typhosum* at a considerably lower concentration than that used for the precipitation of the antigen of *Bact. dysenteriæ* (Shiga). The acetone fractions described in this paper have been obtained by precipitation from an aqueous solution of the antigen with acetone between the levels of 33 to 50 p.c. by volume. In a like manner, the alcohol fractions were precipitated within the limits of 50 to 66 p.c. of alcohol by volume.

Antigenicity tests.—A group of from 2 to 8 rabbits, each weighing 2000–3000 gm., was used for testing the antigenic activity of each preparation. A sample of normal serum was first collected from each animal ; the test material was given intravenously in from 4 to 6 doses each separated by an interval of 2 or 3 days. A sample bleeding was taken 6 or 7 days after the last injection of vaccine ; when necessary, the course of immunization was repeated.

The estimation in vitro *of the O and Vi antibody in the sera.*—Agglutination and agglutinin absorption tests alone were used. Fresh saline suspensions of living organisms prepared from 18- to 24-hour cultures on a trypsin-digest agar were adjusted to such a density that one drop added to the serum dilution contained in 1·0 c.c. NaCl gave the required opacity. Readings were taken

after incubation for 2 hours at 37° C. and 24 hours at room temperature. The strain " Watson " was used exclusively for the estimation of Vi antibody and the strain O " 901 " for the O antibody content. When the sera contained both Vi and O antibody the former was estimated only after complete absorption of O antibody.

Protection tests.—Mice of mixed breed weighing 18–22 gm. were used. In passive protection tests the serum was given intravenously 18 to 24 hours previous to the test-dose of living organisms. In experiments on active immunization the mice received the antigen intravenously in 2 or more doses each separated by an interval of 7 days and the test-dose was given 9 to 14 days after the last injection of vaccine. For both types of experiment the test-dose contained organisms from an 18- to 24-hour agar culture diluted to the required concentration in a trypsin-digest broth ; 0·05 c.c. of the broth dilution of culture was suspended in 0·45 c.c. of a 5 p.c. suspension of mucin (obtained from the Wilson Laboratories, Chicago) and given intraperitoneally. The animals were observed for 7 to 14 days.

The Immunological Properties of the Antigenic Substances Extracted.

(1) *The antigenic material extracted from O strains.*—For this purpose two dried preparations of the strain O " 901 " were extracted by the method described above. These extracts proved to be strongly antigenic ; they produced in rabbits, without exception, O agglutinin titres ranging from 20,000 to 100,000 ; they were also so toxic for rabbits that occasional mishaps occurred and some of the animals died from acute toxæmia within 24 hours. It was hoped that for the purpose of a detailed chemical examination the extracted antigen would prove to be free from Vi as well as H substance. It was shown, however, that whereas H substance was apparently absent traces of Vi substance were present in amounts sufficient to produce Vi agglutinin titres ranging from 1 : 20 to 1 : 100 in a few rabbits.

Partially purified fractions of these extracts were obtained without loss of antigenicity by the precipitation methods detailed above. The dry-weight content of these fractions was accurately determined and it was shown that 3 or 4 injections of 0·25 mg. into rabbits produced sera with O agglutinin titres ranging from 5,000–50,000.

Three horses were immunized with the purified extracts. One animal received approximately 26 mg. of an acetone-precipitated fraction in 8 intravenous injections ; the resulting titre of O antibody was 1 : 50,000. The other horses had previously received Vi extracts and mixed Vi plus O extracts to the point where their O agglutinin titres were 1 : 5,000 and 1 : 10,000 respectively. Each then received approximately 60 mg. of an acetone and alcohol-purified O extract in 6 intravenous doses and the resulting O antibody titres both reached 1 : 100,000. The reaction of the horses to injections was much less marked than our experience in the immunization of horses with other types of typhoid vaccine had led us to expect.

These purified fractions were also examined in precipitation tests with pure O and pure Vi sera. Table I summarizes the results of a test with various antigenic substances and normal and immune sera. It is seen from the Table

that an acetone precipitated fraction of O substance tested in amounts ranging from 1·0 mg. to 0·001 mg. gave strong precipitation with a pure O serum. This preparation also contained distinct traces of Vi substance. One mg. of the fraction in the presence of a pure Vi serum produced definite precipitation ; a finding which is in accord with the results of antigenicity tests in rabbits.

TABLE I.—*Precipitation Tests.*

Extract.		Serum (0·1 c.c. per tube).		
Type.	mg. per tube.	Pure Vi.	Pure O.	Normal rabbit.
O "901" (O) 33–50% acetone fraction	1·0	±	+++±	—
	0·1	(±)	+++	—
	0·01	((±))	+±	—
	0·001	—	±	—
T6S (Vi) 33–50% acetone fraction	1·0	+++	—	±
	0·1	+++	—	—
	0·01	++	—	—
	0·001	((±))	—	—
Ty 2 (O + Vi) 33–50% acetone fraction	1·0	++	+++	—
	0·1	+	+++	—
	0·01	(±)	+±	—
	0·001	((±))	±	—

Total volume per tube adjusted to 1·0 c.c., with 0·85 p.c. NaCL.
Reading taken after 24 hours (1 hour at 37° C. and 24 hours at ± 1° C.).
+++ to ± degrees of precipitation observed with the naked eye.
(±) to ((±)) traces recorded with a hand-lens.

The toxic activity of the O substance was measured by the titration in mice (18–22 gm.) of an acetone-precipitated fraction. Known amounts were diluted with 0·85 p.c. NaCL so that each dose was contained in 0·5 c.c. The mice were injected intravenously and were observed for 3 days. The results obtained with this fraction and others to be described later, are summarized in Table II and it is seen that the average lethal dose of the purified O substance was about 0·1 mg.

TABLE II.—*Toxicity Tests in Mice.*

Extract.		Mice.	
Type.	i.v. dose (mg.).	Tested.	Survived.
O "901" (O) 33–50% acetone fraction	1·0	3	0
	0·25	7	0
	0·1	10	7
T6S (Vi) 33–50% acetone fraction	5·0	10	8
	2·5	5	5
	1·0	3	3
Ty 2 (O + Vi) 33–50% acetone fraction	1·0	3	0
	0·25	7	0
	0·1	10	3

The test-dose in each case was adjusted to 0·5 c.c. with 0·85 p.c. NaCL and given intravenously.

(2) *The antigenic material extracted from rough Vi strains.*—Felix and Pitt (1935) have described strains of *Bact. typhosum* which possess Vi antigen but are apparently completely deficient in O antigen—the so-called " rough Vi " forms. Two such strains were selected for attempts to isolate Vi antigen. One of these (" Ty 441 ") has been described by Felix and Pitt (1935) and the other (" T6S ") was subsequently isolated by Felix and Petrie (1938) as a variant of " Ty 441 " which formed stable suspensions in salt solution and was particularly rich in Vi antigen.

Four dried preparations of " T6S " and two of " Ty 441 " were extracted. Antigenicity tests with the crude extracts showed that they contained Vi substance in an active form. Of 16 rabbits tested with extracts of the strain " T6S " 12 produced Vi antibody ranging in titre from 1 : 100 to 1 : 2000 after 1 to 3 courses of immunization ; the 4 which did not respond received one particular extract and had in all 5 courses of immunization. Extracts of the strain " Ty 441 " produced only an irregular and poor response, for of 10 rabbits which received 1 to 3 courses of immunization with these extracts, only 3 gave Vi antibody in a titre not greater than 1 : 100. In contrast with the lack of potency exhibited by some of these extracts the washed, living organisms before extraction produced in rabbits Vi agglutinin titres ranging from 1 : 500 to the exceptional figure of 1 : 2,000.

Six horses were immunized with " T6S " extracts. Each of four received a total of 25 c.c. extract in six intravenous injections and none produced Vi antibody. The two remaining horses each received a total of 143 c.c. extract in 8 intravenous injections and both gave serum containing Vi antibody in titres of 1 : 200 and 1 : 500 respectively. Dry-weight figures for the extracts used are not available and it is, therefore, not possible to state precisely the amounts of antigen given, but from the dry-weight figures obtained for an acetone-precipitated fraction from these extracts it may be estimated that each of the first four horses received 60 or 70 mg. of substance and that the two which produced Vi antibody each received 300 to 400 mg.

Living suspensions and dried bacterial substance as well as four of the six extracts of the two strains " T6S " and " Ty 441 " failed to stimulate O antibody production in rabbits even after prolonged immunization. Two extracts of the strain " T6S ", however, were shown by antigenicity tests to contain O substance. These two extracts were prepared early in the investigation and we are inclined to the belief that the content of O antigen was due to an oversight in the use of filter-candles through which O extracts had already been passed.

The strain " T6S " is feebly motile and maintenance on dried agar keeps H antigen production at a minimum. As a result, living suspensions as well as the dried substance of this strain, produced H antibody only irregularly in rabbits and of the 4 extracts of this strain only one stimulated H antibody production. On the other hand, both the dried substance and extracts of " Ty 441 " were shown to contain considerable amounts of H antigen. We do not know whether the H substance is soluble in diethylene-glycol or whether flagellar debris passed through the bacterial filter used in separating the glycol extract from the bacterial bodies; the latter suggestion is the more probable one.

Three extracts obtained from the strain " T6S " were purified by the

acetone-precipitation method. These fractions were shown to be antigenically active. A total of 10 mg. injected intravenously into rabbits in 4 or 5 doses produced Vi antibody with regularity in titres ranging from 1 : 200 to 1 : 1000.

The lack of toxicity shown by Vi extracts forms a striking contrast with the high toxicity of O substance, for rabbits could be given 2·0 c.c. of a concentrated extract without reaction and a dose of 6·0 mg. of a purified fraction was also without effect. Reference to Table II, in which the relative toxicity of different fractions for mice is summarized, shows that 5·0 mg. of acetone-purified Vi substance was relatively non-toxic, whereas one-fiftieth of this amount of O substance was an average lethal dose.

The specific nature of the extracted Vi substance and the apparent absence of O antigen from acetone-precipitated fractions is demonstrated in Table I where it is seen that in precipitation tests with normal and type-specific sera amounts of Vi substance ranging from 1·0–0·01 mg. give marked precipitation with pure Vi serum and no trace of reaction with a pure O serum.

(3) *The antigenic material extracted from O + Vi strains.*—The O inagglutinable and virulent strain " Ty 2 " was selected for this purpose. Two dried preparations of the organisms were extracted. Eight rabbits were immunized with extracts from the first preparation and only one responded after 3 courses of immunization by producing Vi antibody with an agglutinin titre of about 1 : 100. Pooled extracts from the second preparation were tested in 5 rabbits all of which produced Vi antibody ranging in titre from 1 : 200 to 1 : 1000. On the other hand, all the rabbits in both series produced O antibody with titres from 1 : 5,000 to 1 : 10,000 after one course of immunization.

It was considered of interest to examine for antigenicity the bacterial residue remaining after multiple extractions with diethyleneglycol and also to examine in a similar manner the final extracts obtained. In one instance, therefore, the bacterial residue after 4 consecutive extractions was tested and also the fourth extract. The residue was prepared for use by washing in diethylene-glycol and resuspending in 0·85 p.c. NaCL. The extract was concentrated approximately 10 times by distillation *in vacuo* at a temperature not greater than 30° C. Each of 3 rabbits receiving the extract produced Vi antibody in titres of 1 : 500 and one of 2 rabbits immunized with the residue gave a Vi titre of 1 : 500 ; data which indicate that the extraction of antigen was far from being complete after four treatments with diethylene-glycol.

Acetone-precipitated fractions were prepared from two " Ty 2 " extracts and the pooled products were tested for antigenicity in 3 rabbits each animal responding with the production of sera with Vi titres from 1 : 100 to 1 : 200, O titres of 1 : 5,000 to 1 : 10,000 and H titres of 1 : 10,000. From experience with " T6S " preparations the production of H antibody in this case was not surprising. The strain " Ty 2 " is rich in H antigen and all the rabbits immunized with extracts of this strain gave sera with H titres from 1 : 1,000 to 1 : 10,000.

The toxicity of " Ty 2 " extracts and the purified fractions obtained from them closely paralleled that of the O substance. Table II shows that the average lethal dose of the acetone-precipitated fractions of " Ty 2 " for mice is similar to that of the O fraction, namely, 0·1 mg.

Precipitation tests with " Ty 2 " preparations gave the expected result.

Reference to Table I shows that 0·001 mg. of the acetone-purified fraction gave distinct precipitation with a pure O serum; a finding which is in agreement with the results obtained in toxicity tests. It is also seen from the Table that 0·1 mg. of the "Ty 2" preparation produced a positive result with a pure Vi serum whilst the "pure" Vi preparation, "T6S", reacted positively in amounts of 0·01 mg.; the presence of O substance in the "Ty 2" preparation and a lower concentration of Vi substance may account for this difference.

The Chemical Properties of the Antigenic Fractions.

Aqueous solutions of the antigenic preparations that are of approximately equal concentration and that have been obtained from O, O + Vi and rough Vi strains of *Bact. typhosum* in the manner described are found to be of equal opalescence. It has been observed, however, that the opalescence of the preparations decreases during the purification and fractionation processes, especially if these are carried out at room temperature and not at 0° C.

The antigenic material, irrespective of the specific immunological condition of the strain employed, was ultimately obtained as a white amorphous solid by rapid evaporation, at low temperatures and *in vacuo* of the frozen aqueous solution. When prepared in this manner it readily redisperses and yields a stable suspension in water and saline. It has been observed that these colloidal solutions, when agitated, show a shimmer similar to that which is characteristic of suspensions of long thread-like molecules. This interesting optical property can be considerably reduced and sometimes completely removed if the aqueous solution of the antigenic preparation is filtered through a British Berkefeld filter-candle.

A number of qualitative tests have been applied to the different antigen preparations and in some instances a distinct difference in reactivity has been recorded. 0·5 to 1·0 p.c. solutions of the active fractions were treated with equal volumes of the following chemical reagents: Uranium acetate, mercuric acetate, phosphotungstic acid and aluminium sulphate. The results are of considerable interest for it has been observed that in antigenic preparations made from the strain O "901" none of these reagents produced a precipitate, whereas, when antigenic material that has been obtained from the Vi-containing strains, "Ty 2" and "T6S" is employed, a precipitate is formed. Similar results have been recorded by Topley *et al* (1937). We have observed, however, that those Vi preparations that are free, or practically free, from O antigen precipitate immediately with these reagents whereas O + Vi preparations usually require to stand several hours before precipitation is complete and the supernatant fluid is quite clear. Our Vi-containing preparations have failed to give more than a trace of precipitate with neutral lead acetate; the O antigenic substance from the strain O "901" gives no precipitate with this reagent. Furthermore, it has been found that the material from the rough Vi-containing strain, "T6S", fails to yield a positive biuret test and the O + Vi substance gives only a weak reaction compared with the strongly positive test given by all of the O antigenic fractions that have been obtained from strain O "901". Similarly, the ninhydrin tests on O-containing material are strongly positive, whereas, substances obtained from the strain "T6S"

that contain Vi antigen and are free from O antigen fail to give a positive ninhydrin test. All preparations fail to precipitate with trichloracetic acid, Esbach's reagent or salicylsulphonic acid; protein, therefore, appears to be absent. Tests for the hexuronic acids, pentoses and ketoses were negative but diazobenzenesulphonic acid gave a strong reddish-yellow colouration with the antigenic material from strain O " 901 ", whereas, the O + Vi-containing substance from the strain " Ty 2 " gave a very much weaker test and " pure " Vi material from the rough strain " T6S " gave only a pure yellow colouration of equal intensity and tint with the control diazobenzenesulphonic acid in *N* sodium carbonate. Glycogen and serologically active carbohydrate-like material derived from agar appeared to be absent from all of the antigenic fractions.

A few analyses of the various antigens after purification by treatment with acetone or alcohol have given the following analytical figures which are stated as percentages. Preparations from the strain O " 901 ", C 46·1, H 7·6, N 2·6, P 1·7, ash 11·7, from the strain " Ty 2 ", C 48·7, H 8·2, N 2·5, P 2·1, ash 11·7, and from the strain " T6S ", C ?, H ?, N 3·6, P 1·7. These are typical analytical results but slightly different figures were sometimes obtained with individual preparations. All the extracts we have examined contained sulphur, but the form in which it is present has not yet been determined.

Passive and Active Protection Experiments.

For the purpose of examining the protective action of the various Vi and O immune-sera produced in rabbits their therapeutic value was estimated by a mouse protection test the technique of which has been described above. In these tests, we have confirmed the findings of Rake (1935), Siler (1936) and Buttle *et al.* (1937) with regard to the value of mucin as an adjuvant to the test dose of typhoid bacilli when given intraperitoneally. For example, the strain " Ty 2 " which possesses both Vi and O antigen and is known to be fully virulent for mice was found to have an average lethal dose (killing 50–80 p.c. of mice) of 50 million organisms when diluted in a trypsin-digest broth and given in 0·5 c.c. amounts; when suspended in mucin, however, the average lethal dose of the same culture, contained about 500 organisms. This technique has given regular results and has brought to light several points of interest concerning the relation of the protective action of a serum to the " virulence " of the invading organism. This subject, however, will be discussed in another paper.

Table III summarizes the results of a protection experiment in which the value of sera prepared respectively by immunizing with the purified O substance, the purified Vi substance, dried rough Vi bacilli and living rough Vi bacilli have been compared. A group of control mice which received normal rabbit serum were included in the experiment and the suspension of the test strain " Ty 2 " was titrated in these mice. There were approximately 500 organisms in the average lethal dose and all mice receiving the sera under test were given approximately 100 average lethal doses. The animals were observed for 10 days; deaths occurred mostly within 24 to 72 hours, but in those mice receiving the smaller doses of serum occasional deaths occurred up to the 7th day. The spleen of mice dying after the 3rd day was examined

culturally and *Bact. typhosum* was isolated from 10 out of 11 mice. The surviving and apparently healthy mice which had been given the smallest dose of each serum were killed on the 10th day and the spleens examined culturally. *Bact. typhosum* was isolated from approximately 50 p.c. of these mice irrespective of the serum group from which they were taken. This result was to be expected because, in other experiments, where much larger amounts of the same sera were used, a small proportion of apparently healthy mice were found to be carrying *Bact. typhosum* in the spleen 12 days after the test-dose had been given.

The protective efficiency of a pure O serum is clearly demonstrated in Table III ; 0·001 c.c. of a serum with an O agglutinin titre of 1 : 50,000 and a Vi titre of less than 1 : 10 protected 4 of the 10 mice and larger doses of the serum protected a higher proportion of animals. In other experiments, however, it was shown that the value of this serum rapidly diminished when the test-dose of organisms was increased. Nevertheless, the result shown in Table III forms a striking contrast to those recorded by Felix and Pitt (1934*b*). They found that 1·0 c.c. of an O serum with an agglutinin titre similar to the one used in the present experiment was entirely without effect against infection with the strain " Ty 2 ". A possible explanation of this discrepancy will be discussed in a subsequent paper and may be found to lie in differences in the methods of test.

TABLE III.—*Passive Protection Tests in Mice.*

Serum. From rabbits immunized with antigens.	Agglutinin titre. Vi.	Agglutinin titre. O.	i.v. dose (c.c.).	Mice. Tested.	Mice. Survived.	Number of organisms per dose.
O (33–50% acetone fraction)	<1 : 10	1 : 50,000	0·005	10	9	50,000
			0·002	10	6	
			0·001	10	4	
Vi (A) (living organisms)	1 : 2,000	1 : 50	0·005	10	10	50,000
			0·002	10	9	
			0·001	10	5	
Vi (B) (33–50% acetone fraction)	1 : 2,000	1 : 100	0·05	10	8	50,000
			0·02	10	5	
			0·01	10	1	
Vi (C) (acetone-dried organisms)	1 : 10,000	<1 : 10	0·002	10	10	50,000
			0·001	10	9	
			0·0005	10	4	
Normal rabbit serum	<1 : 10	1 : 100	0·05	10	0	50,000
				10	0	5,000
				10	2	500

Test-dose prepared from the strain Ty 2.

Table III also shows the relative protective value of 3 pure Vi sera (A, B, C). Serum A was prepared by immunizing rabbits with living rough Vi bacilli ; serum B with extracted Vi antigen and serum C with acetone-dried rough Vi bacilli before extraction. Sera A and B had the same Vi agglutinin value (1 : 2000) ; serum C had a Vi agglutinin titre of 1 : 10,000. On the assumption that the Vi agglutinin content reflects protective value, sera A and B should have given the same result *in vivo* and serum C should have been approximately 5 times stronger. The results recorded in Table III, however, show that the

value of serum B was 20 to 50 times lower than that of serum A, while serum C was at least twice as potent as A. The reasons for this functional deficiency of serum B are not known, but it has been observed with regularity and appears to be a characteristic of Vi antibody produced by immunizing with all the diethylene-glycol extracts of the rough Vi strain "T6S" that we have tested. It was thought that the process of acetone drying before extraction might in part be responsible, but a comparison of the protective values of sera A and C, which are shown in Table III, indicates that the influence of acetone is slight. It is more probable that the process of extraction renders the Vi substance deficient in respect of certain groupings on the antigen molecule which may be responsible for the production of protective immune-body in the rabbit while leaving unimpaired those groupings which are responsible for agglutinin production. The examination of Vi antibody produced in different species of animals may illuminate this point. On the other hand, it may be that the untreated bacterial cells possess an antigen which has remained undetected; one which is of importance in the production of protective immune-body and is relatively insoluble in diethylene-glycol.

The antigenic action of the various kinds of extracts has been tested in mouse experiments. Table IV summarizes the results of an experiment in which the activity of Vi, O and O + Vi acetone-purified extracts was estimated. For each preparation two groups of mice were included; one group received 0·01 mg. of antigen per dose and the other 0·001 mg. The experiment also included a group of mice given a non-specific stimulus in the form of an acetone-purified extract of *Bact. dysenteriæ* (Shiga). All mice received 2 intravenous doses of the respective antigens with a 7-day interval between the doses. They were tested 10 days after the last injection of vaccine with the virulent O + Vi strain "Ty 2" given intraperitoneally as described above. The test suspension was titrated in a group of non-vaccinated controls and reference to Table IV shows that all vaccinated mice received approximately 100 average lethal doses. The animals were observed for 10 days; deaths occurred over a period of 1 to 6 days.

TABLE IV.—*Active Immunization Tests in Mice.*

Extract.		Mice.		Number of organisms per dose.
Type.	i.v. dose (mg.).	Tested.	Survived.	
O (33–50% acetone fraction)	0·01	10	8	50,000
	0·001	10	7	
Vi (33–50% acetone fraction)	0·01	10	9	50,000
	0·001	10	7	
O + Vi (33–50% acetone fraction)	0·01	5	5	50,000
	0·001	10	9	
Bact. dysenteriæ (Shiga) (50–66% acetone fraction)	0·01	20	3	50,000
Nil.	..	20	1	50,000
		20	7	500

Test-dose prepared from the strain Ty 2.

Table IV shows that the non-specific antigen was not entirely without effect; 3 out of 20 mice survived the test-dose. This result, however, does not minimize the activity of the specific typhoid antigens. The value of O-extract is clearly demonstrated; a finding which corroborates that of Topley *et al.* (1937) but is not in accord with that of Felix and Pitt (1934*b*) and the more recent work of Kauffmann (1937). As suggested in the section dealing with passive protection an explanation of this discordance is probably to be found in the methods of test employed by different workers. The pure Vi extract is seen to have induced a degree of immunity similar to that of the O extract and the O + Vi extract appears to be slightly better than either of these. Further experiments concerning the possible functional defects of Vi extracts require to be undertaken using larger groups of animals and a wider range of doses of the antigens.

DISCUSSION.

The extraction of dried cultures of *Bact. typhosum* with anhydrous diethylene-glycol has yielded substances which are antigenically active and which appear to be free from protein. This finding is in agreement with that of Mesrobeanu (1936) who used trichloracetic acid for extraction and with that of Topley and his co-workers (1937) who digested the bacteria by means of trypsin. Moreover, the chemical nature of the antigenic substances that we have obtained appears to resemble in many respects the material isolated from *Bact. typhosum* by these workers. There are, however, some differences in chemical composition to be recorded. The antigenic material isolated by Topley *et al.* from an O + Vi strain contains a considerably higher content of nitrogen (4·8–5·2 p.c.) than the substance we have isolated (2·25–2·4 p.c.). Similarly, Topley *et al.* found 4·9–5·1 p.c. of nitrogen in their preparations from an O strain, whereas, we have found 2·5–2·8 p.c. in the substance obtained from a similar strain; this result is, however, in close agreement with the data recorded by Mesrobeanu, whose material contained 2·47 p.c. of nitrogen. Furthermore, the phosphorus content of her material is given as 1·45 p.c., which agrees closely with that found for the antigenic material separated by us from the strain O " 901 "; the acetone fraction of this preparation contained 1·6 p.c. of phosphorus. On the other hand, we have found the phosphorus content of the substance from O + Vi strains to be somewhat higher, 2·0–2·2 p.c. and the antigenic material from the rough Vi strains to have an intermediate value of 1·7–1·8 p.c. With the small quantities of antigenic material at our disposal it has not been possible to carry out satisfactory fractionations and thus ascertain whether the material under investigation was entirely homogeneous.

Our experiments have given information concerning the identification of the Vi antigen as a separate chemical entity. The detection of Vi antibody in the serum of animals immunized with certain strains of *Bact. typhosum* led Felix and Pitt (1934*b*) and other workers to conclude that Vi substance exists in the bacterial cell as a complete antigen which is separate and distinct from the O and H antigens. Topley and his colleagues (1937) accept this as a possibility but suggest an alternative hypothesis. From the results of experiments on active immunization, they speculate that O and Vi antibodies may be

produced by one basic antigen, namely the O substance which under certain conditions acquires labile chemical groupings that stimulate the production of the Vi antibody. These workers agree, however, that this hypothesis is rendered less probable by the more recent report of Felix and Pitt (1935) concerning the strains of *Bact. typhosum* from which the O substance appears to be absent, but which stimulate the production of and absorb the Vi antibody. Our results corroborate those of Felix and Pitt. We have isolated from their so-called rough Vi strains material containing Vi substance which is antigenically active, and which is free from antigenically active O substance; this substance also exhibits chemical and immunological properties that differentiate it sharply from the extracts containing O antigen. On the other hand, the O substance is easily extracted from both O and O + Vi strains, but we have not succeeded in isolating it in a form entirely free from Vi antigen.

An interesting problem concerning the chemical nature and immunological function of the Vi substance as it exists in the untreated bacterial cell and as it may be modified by simple chemical treatment has emerged from the results of our experiments on passive protection. We have obtained various pure Vi sera in which the agglutinin titres and protective values do not correspond. Thus, a pure Vi serum prepared by immunizing rabbits with diethylene-glycol extracts has a protective value which is significantly lower than that of a Vi serum prepared by immunizing with a living suspension of the rough Vi strains. Our experience in this respect, however, is not unique; a similar phenomenon was recorded by Felix and Bhatnagar (1935) with Vi sera prepared by immunizing with formolized extracts of *Bact. typhosum*. Further, the observations of Avery and Goebel (1933) on the acetylated and deacetylated polysaccharide of the pneumococcus and those of Topley *et al.* (1936) on substances isolated from *Bact. œrtrycke*, may be cited as other examples although, in these cases, the alteration in functional activity is generally characterized by a loss of agglutinogenic and precipitinogenic activity while retaining the power to stimulate protective immune body. In our experience and that of Felix and Bhatnagar (1935) the modified antigen produces agglutinins and precipitins but its value as a protective antibody is greatly diminished. The reasons for this type of deficiency are not known, but at least two explanations can be suggested. The first is that the untreated bacterial cell of *Bact. typhosum* contains an antigen which has so far remained undetected; one which is of importance in the production of protective immune-body and which is relatively insoluble in diethylene-glycol. The second, and perhaps more probable explanation, is that the treatment of the cells with organic solvents modifies the antigenic complex. We are inclined to the belief that these solvents bring about a gradual change in the physical, chemical and antigenic properties of the immunologically active material.

Finally, the method of extraction with diethylene-glycol which has been used in the present experiments and in those relating to *Bact. dysenteriæ* (Shiga) (Morgan, 1937) may prove of value in the isolation of antigenic substances from other organisms. In some preliminary experiments, however, we have found it to be applicable only to the gram negative bacteria we have tested. For example, we have attempted to isolate without success, antigenic substances from certain gram positive organisms including pneumococcus

Types I and II, *Vibrion septique* and *Cl. welchii*. In each case hapten-like fractions were obtained which gave a specific precipitation reaction with type sera, but which failed to stimulate the production of an antibody demonstrable *in vitro*. These results are in accord with those of Boivin and his colleagues (1937) who failed to isolate an antigenic substance of a non-protein nature from pneumococci and with those of Stamp and Hendry (1937) who have isolated from certain streptococci a protein fraction which has the immunizing properties of the whole bacterial cells. It may be, therefore, that protein forms an essential part of the antigenic complex of those gram-positive organisms that we have tested so far.

SUMMARY.

The extraction of suitable strains of *Bact. typhosum* with anhydrous diethylene-glycol yields substances which are antigenically active and which are apparently free from protein.

The Vi antigen can be detected in extracts from rough Vi strains as a chemical entity separate and distinct from O substance.

Substances containing O antigen are readily isolated from the O agglutinable strains, but we have not succeeded in obtaining these preparations entirely free from Vi antigen.

Traces of H antigen have been detected in the extracts obtained from strains that are rich in flagella. This is probably due to flagellar debris which passes through the bacterial filters used in the preparation of the extracts.

The functional activity of the antigenic complex in rough Vi strains is modified by the process of extraction. The immune-body produced by immunizing with these extracts is much less effective in protecting experimental animals than that produced by immunizing with living suspensions of rough Vi bacilli.

The value of mucin as an adjuvant to the test-dose of typhoid bacilli for infection experiments in mice has been confirmed.

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